University of Alberta

Target Molecules for Reactive Free Radical Metabolites of Aromatic Amines

BY

Malyaj Narwaley

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ABSTRACT

Aromatic amines during peroxidase metabolism produce N-centered cationic radicals and phenyl radicals as reactive intermediates. The latter also induce protein oxidation. These findings are associated with drug induced agranulocytosis. However, other biomolecular targets of these metabolites are unknown. We tested the reactivity of aromatic amines and congeners with selected poly-unsaturated fatty acids (PUFA) and glutathione (GSH) in the presence of horseradish peroxidase (HRP)/H_2O_2 by Oxygen electrode, Electron spin resonance (ESR), HPLC and immunospin trapping. Our results show that aromatic amines generating phenyl radical metabolites in presence of HRP/H_2O_2 oxidise PUFA to form lipid peroxides but do not oxidise GSH. Aromatic amines which do not form detectable phenyl radical oxidized only GSH. Furthermore, ESR and gel-electrophoresis studies showed that PUFAs are target for phenyl radical metabolite and scavenge them, and prevent the protein oxidation induced by phenyl radical. In conclusion, these results suggest a possible mechanism for aromatic amine induced agranulocytosis.

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LIST OF ABBREVIATIONS:

AG	Aminoglutethimide
AB	Amino benzene
BSA	Bovine serum albumin
DAB	N,N-dimethyl-4-aminobenzene
DHA	Docosahexaenoic acid
DMPO	5,5-dimethyl-1-pyrroline-*N*-oxide
DNPH	2,33-dinitrophenyl hydrazine
ESR	Electron spin resonance
EPR	Electron paramagnetic resonance
GADPH	glyceraldehyde 3-phosphate dehydrogenase
GO	Glucose oxidase
GSH	Glutathione
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
HPLC	High performance liquid chromatography
IDA	Idiosyncratic drug induced agranulocytosis
IDR	Idiosyncratic drug reaction
LA	Linoleic acid
MAB	Monomethyl amino benzene
MNP	2-methyl-2-nitrosoprapane
MPO	Myeloperoxidase
MDA	Malondialdehyde
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
PAN	*para*-anisidine

PB	Phosphate buffer
PBS	Phosphate buffer saline
PUFA	Polyunsaturated fatty acid
RT	Room temperature
SA	Sorbic acid
SDS	Sodium dodecyl sulfate
TCEP	Tris-(2-carboxyethyl) phosphine
TBS	Tris base saline
TMP	1,1,3,3-trimethoxypropane
UV	Ultra violet

Chapter 1

Introduction

1.1 Sources of Aromatic amines and toxicities

Exposure of humans with aromatic amines and their toxicological side effects has been historically significant in toxicological studies. Directly or indirectly, human beings are potentially exposed to aromatic amines continuously. Aromatic amines are commonly present in industrial wastes, intermediates of polymer production, dyes, pesticides, xenobiotics, environmental pollution and tobacco smoke (*1*). Their presence is also commonly reported in dietary components of broiled beefsteaks and broiled fish (*2*).

These aromatic amines have been associated with severe and lethal toxicities since early times. In 1930, Japanese scientists were first to show hepatocarcinogenic effects of pure aromatic amino-azo dyes in rats and mice (*3*). Miller and co-workers in 1943, started a study on N, N-dimethyl-4-aminoazobenzene (DAB) and its carcinogenic effects in rats and mice liver. When DAB was given for several months to rats at 0.06% (6000 µg DAB/rat/day) in a diet low in riboflavin, a high yield of multiple tumors in liver was observed (*3*) and was attributed to very low metabolism of DAB in liver. They also were able to isolate demethylated metabolites of DAB which were also azo-dyes, namely N-monomethylaminoazobenzene (MAB), 4-aminoazobenzene (AB). Out of these, MAB and DAB were found to be having equal carcinogenic activity, while AB was suggested to be non-carcinogenic. Further in 1947, Miller and Miller tested a

series of azo-dyes and suggested that presence of at least one N-methyl group was necessary for producing the carcinogenic effect by azo-dyes or their metabolites (Figure 1.1) (*3*, *4*). A protein bound water soluble 3-(homocystein-*S*-yl)-MAB was confirmed which is suggested to breakdown *in vivo* into further into non-polar dye 3-methylmercepto-MAB (*5*)

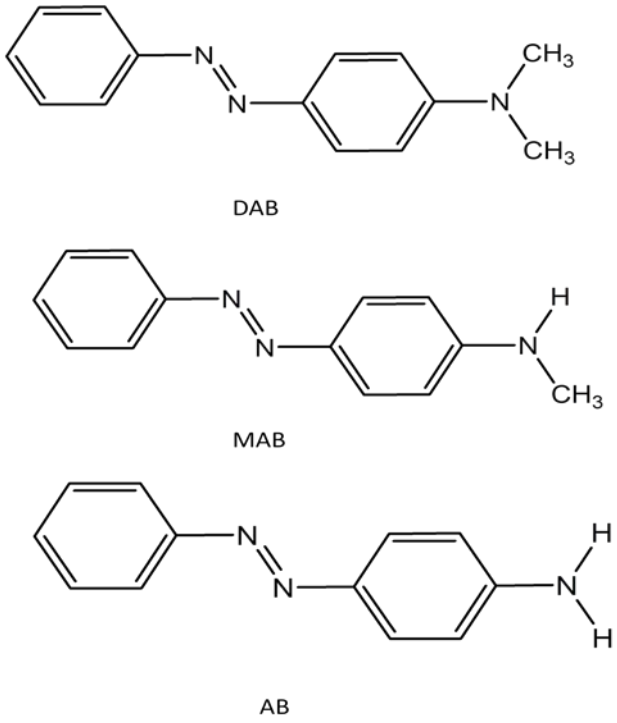


Figure 1.1 Structures of carcinogenic aromatic amine azo dyes

N,N-dimethyl-4-aminoazobenzene (DAB), N-methyl-4-aminoazobenzene (MAB) and aminoazobenzene (AB).

In the late 19th century, a skilled clinician came up with an observation in a dye industry German Rhineland that number of employees presented with urinary bladder cancer. These pioneering findings were later studied on workers

of dyestuff industry in Great Britain (*6*). 4-Aminobiphenyl and benzidine which are used in the dye industry have been suggested to be associated with urinary bladder cancer (*6*). Furthermore, the presence of 4-aminobiphenyl is also reported in tobacco smoke and is associated with urinary bladder carcinoma along with its other side effects like heart and lung diseases (*7*).

In the past, the aniline workers were called as “blue boys” due to aniline toxicity and causing formation of ferrihemoglobin (methemoglobin). Some lifesaving aromatic amine xenobiotics in past days were marketed for some time like sulfanilamide, which caused formation of ferrihemoglobin. Further, it was investigated and was suggested that it is the aromatic amines which are most frequently responsible for the formation of ferrihemoglobin *in vivo*. However, except for diamines and aminophenols, aromatic amines have to undergo metabolism and produce active compounds to react with hemoglobin of blood. It was suggested that amongst the different derivatives, N-hydroxy derivatives of aromatic amines were more important in ferrihemoglobin formation *in vivo*. Further, this metabolite was also correlated with the carcinogenic effects of aromatic amines. Keeping in mind the other active compounds like N-oxides (also responsible for ferrihemoglobin formation) and nitroso compounds, which are frequently attributed to carcinogenic activity, it was suggested that it seems that it is same “active structures” of the nitrogen which are involved in both cases (*8*).

Apart from carcinogenic and hematological toxicity, several aryl amines have been associated with drug induced lupus, which is a drug induced

autoimmune syndrome suggested to involve antigen-antibody complex formation (*9*). Aromatic amine drugs like sulfonamide, aminosalicylate, and procainamide have known to cause idiosyncratic hepatic damage. The drug, procainamide drug has been reported to cause lupus incidence as high as 30%. Other aryl amines which are also reported to induce lupus are nomifensine, aminoglutethimide. Interestingly, the non-aromatic amine drugs like proctolol and acetolol are also responsible for lupus. Though they don't fall under the category of aromatic amines, but they are known to be metabolized extensively into aryl amines *in vivo* (*9*). It is widely suggested that, the metabolites of procainamide and other aryl amines formed *in vivo*, are responsible for the idiosyncratic drug reactions like lupus (*9, 10*).

1.1.1 Role of aromatic amines in drug induced agranulocytosis

Many of the aromatic amine drugs causing lupus are also associated with agranulocytosis (*9, 11*). Drugs like aminoglutethimide, procainamide (*9-11*), dapsone (*9*), sulfonamide (*10*) and many related aromatic amines and their congeners (*11*) are associated with agranulocytosis toxicity. The latter is a condition which is characterized by a decrease in neutrophil counts (<500/µl of blood) (*11*). In a majority of patients the neutrophil count is <100/µl (*12*). Agranulocytosis is an idiosyncratic drug reaction like lupus, which means specific to an individual; thus an idiosyncratic drug reaction is an adverse reaction that does not occur in most people within the range of doses used clinically (discussed

in more detail in chapter 1.3) (*13*). The criteria for drug potency to cause agranulocytosis is the onset of agranulocytosis within 7 days in the case of intake of the drug previously, and after 1 month of drug interruption, a complete recovery with more than 1500/µl neutrophils of total blood cell count. Although there should be a recurrence of agranulocytosis while re-administration of same drug but usually such criterion is not confirmed due to the mortality rate (*12*). The annual incidences of idiosyncratic drug induced agranulocytosis (IDA) in Europe is between 3.4 and 5.3 per million of population, while in USA it is 2.5 to 15.4 per million per year (*12*). IDA is a lethal disorder with a mortality rate of 5-20% (*14*). A cohort study done in Hopitaux Universitaires Strasbourg showed that 70% of the patients suffering from IDA were ≥60 years in age. Only 10% of the cases are reported in young and adults while the incidences of IDA increase with age. Further, the incidences are reported more in woman (almost twice) as compared male patients in humans, which is attributed to increased rate of medication usage by women (*14, 15*). Until 1990, Europe had a high death rate of 10-15% from IDA. This rate recently dropped to <10% possibly due effective treatment and therapeutic strategies of modern times like hematopoietic growth factors (*14, 16*). The highest mortality rate is observed in elderly patients of age >65 years. Also, high mortality is reported in those experiencing renal failure, bacterial infections or septic shock at diagnosis (*12*).

The lowering of neutrophil counts increases the risk of severe and fatal infections such as pneumonia and septic shock in the patient (*17*). Conditions like oropharyngeal, anorectal and skin infections have also been reported. In most of

the patients, hematological parameters like hemoglobin and platelet counts remain normal. However, in elderly patients, it is reported that at least 10% of the patients suffer thrombocytopenia and at least 30% have hemoglobin <120 g/L.

1.2 Aromatic amine and its metabolism

All heterocyclic aromatic amines are suggested to undergo hydroxylation at the nitrogen atom to give an N-hydroxylated amino group by microsomal cytochrome P-450 enzyme (*2*). In the mid-20th century, it was found that the presence of glucose-6-phosphate, NAD and NADPH enhanced the metabolism of DAB (*3*). In the later years, N-hydroxylated metabolites were discovered during *in vivo* studies of rats fed 2-acetylaminoflourene, which is known to have carcinogenic effects (*18*).

Aromatic amines consist of an amino group and an aromatic nucleus which in some is substituted at nitrogen or at aromatic ring. The most basic structure of this class is aniline (aminobenzene). It is suggested that any of these group can be the site of metabolism for aromatic amines. *N*-acetyl transferases (NATs) are known to play major role in aromatic amine metabolism. *O*-acetylation of *N*-hydroxyl amines is also known which is suggested to be mediated by NATs. It is considered as a detoxification process which reduces the oxidation of amino group to N-hydroxy aryl amines by cytochrome P-450 enzymes (*19*). In dogs which does not contain NATs enzymes, in 1960, both *para* and *ortho* hydroxylated metabolites of aniline have been reported (*20*). In rabbit,

during 1948, *para* and *ortho* aminophenols have been isolated which demonstrates the hydroxylation reaction of aniline in microsomes. In rabbits however, it is suggested that aromatic amines undergo acetylation reaction prior to hydroxylation. Further, it has been suggested that for the microsomal metabolism of amine compounds, the presence of reduced NADPH and molecular oxygen was required for hydroxylation. Further, presence of cytochrome P-450 enzyme inhibitor like diethylaminoethyldiphenylpropylacetate hydrochloride (SFK-525A) inhibited the hydroxylation which suggests the involvement of P-450 enzymes in hydroxylation of the aromatic amines. In some metabolic reactions, direct involvement of amino group can be observed leading to formation of N-glucuronic acid or N-sulphates. The former has been isolated in rabbit during administration of dapsone and the latter in case of phenetidine (*20*).

Similar to the aniline, in N-naphthylamine, acetylation is observed in rodent species. It is suggested that, acetylation promotes the oxidation of aromatic ring resulting into formation of 6 and 5,6-hydroxylated product of N-naphthylamine. N-naphthylamine is associated with urinary bladder carcinogenesis (*20*). The formation of N-hydroxy derivative is widely accepted intermediate metabolite having role during toxicities like carcinogenesis, ferrihaemoglobinemia, methemoglobinemia, cellular necrosis and mutagenesis (*1, 8, 18, 20-22*).

Nitrosobenzenes as another group of metabolites have been reported during erythrocytic metabolism of aromatic amines. These are suggested to be formed by further oxidation of hydroxylamines. Hydroxylamines may release

hydroxyl group to form nitrosobenzene and react with thiols of glutathione (Figure 1.2) (*23*). This is proposed to delay the elimination of aromatic amines inside the body may be a cause of toxicities like ferrihemoglobinemia and methemoglobinemia (*20*, *23*).

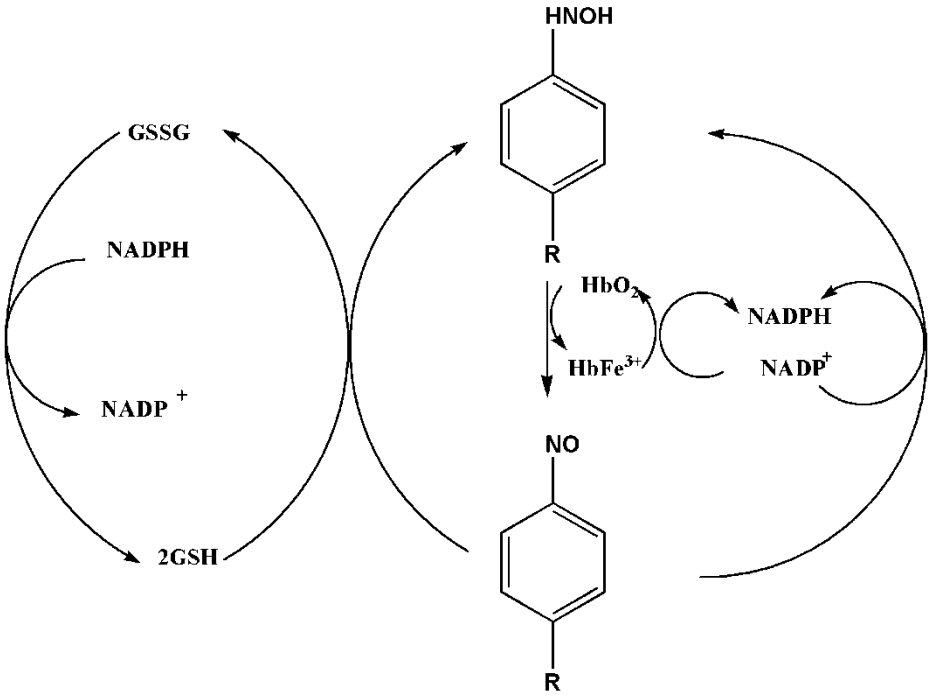


Figure 1.2 Metabolism of aromatic amine in RBC

Metabolism of aromatic amines in red blood cells showing formation of N-hydroxylamine and nitrosobenzene (*23*).

1.2.1 Metabolism of aromatic amines in relation to drug induced agranulocytosis

Formation of reactive metabolites by aromatic amines is also suggested to be one of the initiating factors for drug induced agranulocytosis (*9-12*, *22*). As

agranulocytosis is associated with the depletion of neutrophil count, thus there is high possibility of aromatic amines undergoing enzymatic metabolism by neutrophil enzymes (*9*). Neutrophils contain myeloperoxidase; an enzyme with bactericidal properties that is also capable of drug oxidation. In presence of hydrogen peroxide, myeloperoxidase produces an intermediate compound-I (Figure 1.3). Compound-I is a highly oxidative compound which can oxidize chloride to produce hypochlorous acid. Further, hypochlorous acid is also known to participate in drug metabolism as shown in (Figure 1.4). The hydrogen peroxide needed for this reaction is produced by the action of NADPH oxidase which converts oxygen to superoxide.

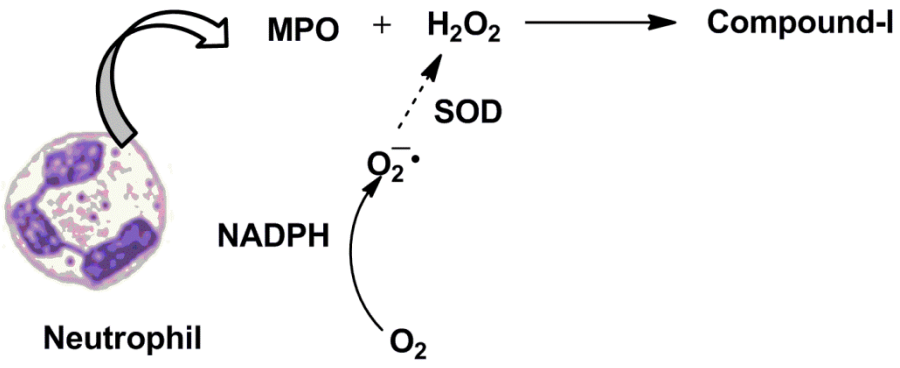


Figure 1.3 Formation of compound-I from MPO

Schematic representation of release of myeloperoxidase (MPO) from neutrophil and formation of compound-I in presence of hydrogen peroxidase, NADPH oxidase and superoixde dismutase (SOD).

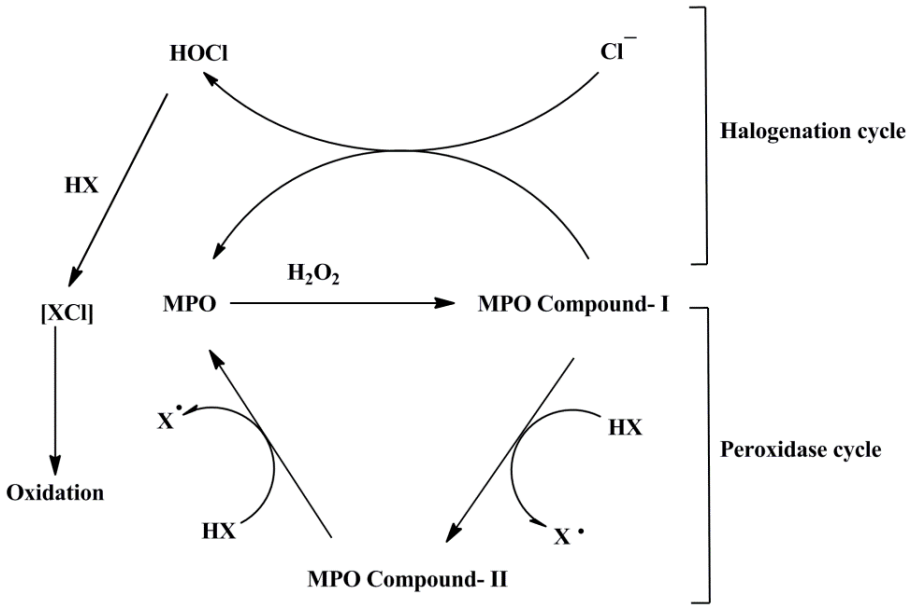


Figure 1.4 Schematic representation of peroxidase cycle and halogenation cycle mediated by

Peroxidase cycle representing formation of hypochlorous acid (HOCl) and oxidation of drug (HX) mediated by compound I from MPO in presence of H_2O_2. HOCl can also oxidize drug (HX) to free radical ($X^{\cdot}$) and form chlorinated reactive metabolite (XCl) which further may undergo oxidation.

Superoxide subsequently forms hydrogen peroxide. Hypochlorous acid thus produced has anti-bacterial properties and helps in protecting against infections (*10*). Although myeloperoxidase is present in azurophilic granules in neutrophils during their resting state, when neutrophils are activated by agent like bacteria, myeloperoxidase is released outside. Interestingly, this myeloperoxidase is released outside the cells and NADPH oxidase is also activated outside the plasma membrane during infection.

Aromatic amines are metabolized in the presence of myeloperoxidase of neutrophils into N-hydroxylamines and further into nitroso compound. The latter

metabolite is known to be more reactive and cause oxidation of glutathione and to form covalent bonds with neutrophils (Figure 1.5) (*10*). However the intermediates in these reactions are free radicals.

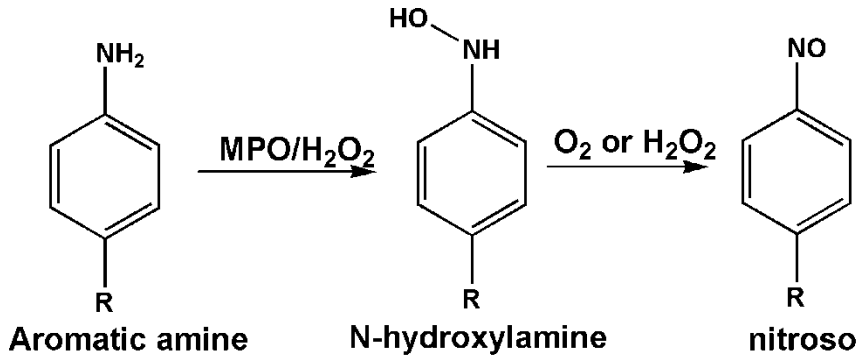


Figure 1. 5 Formation of N-hydroxylamine and nitroso during peroxidase metabolism of aryl amines

Metabolism of aromatic amines catalysed by myeloperoxidase (MPO)/hydrogen peroxide (H_2O_2) and formation of hydroxylamine and nitroso. Latter is proposed to be formed in presence of H_2O_2 or oxygen (O_2).

1.2.2 Role of free radical metabolites in drug induced agranulocytosis

A new approach has been suggested lately which takes under consideration the involvement of free radical metabolites during myeloperoxidase mediated metabolism of aromatic amines (*11, 22, 24*). It has been observed that the aromatic amines undergo peroxidase metabolism to form nitrogen centered cation radicals. A carbon centered phenyl radical has been detected during peroxidase metabolism of aromatic amine followed by nitrogen centered cationic radical *in vitro* (*11, 25, 26*). Until now, series of aromatic amines have been detected to produce phenyl radicals during myeloperoxidase or horseradish peroxidase mediated metabolism, which is suggested to be formed from nitrogen

centered cationic radical intermediate (Figure 1.6). Further, all of these aromatic amines have been associated with drug induced agranulocytosis (*11*).

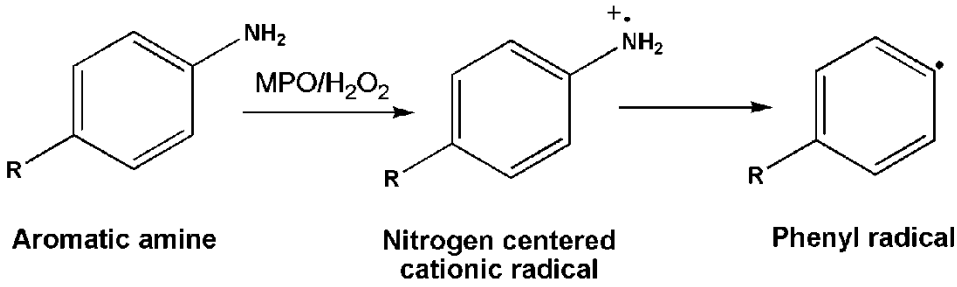


Figure 1.6 Peroxidase metabolism of aryl amine into free radicals

Schematic representation of formation of free radical metabolic intermediates of aromatic amine during peroxidase mediated metabolism.

1.3 Idiosyncratic drug reactions and proposed mechanisms of agranulocytosis

By definition, an idiosyncratic drug reaction (IDR) is an adverse reaction which is specific to a person and does not occur to most people at the clinical dosage range (*13*). For most of the IDRs, it is hard to get clinical data of its incidences because of its individual specific nature. Aromatic amines are associated with idiosyncratic drug reactions frequently. As discussed previously, they are responsible for IDRs like lupus and agranulocytosis. However, the exact mechanism for most of the drug induced idiosyncratic agranulocytosis (IDA) is still unknown. Amongst various suggested mechanisms, an immune mediated response to IDR is frequently discussed in literature (*9, 12, 13*). In case of procainamide, anti-myeloid antibodies have been found in drug induced

agranulocytosis (*9*). Similarly, immune mediated response to aminopyrine during agranulocytosis toxicity is also evident (*10*). During agranulocytosis, destruction of bone marrow cells has also been observed. In some cases, destruction of cells in bone marrow due to immune reaction has also been suggested (*9*). From above discussion regarding aryl amine metabolism mediated by myeloperoxidase it is reasonable to correlate the involvement of reactive intermediates of aromatic amines with neutropenia. While in some cases, these reactive intermediates can themselves act as antigens to trigger immune response to produce anti-neutrophil antibodies (*9*). Further, a direct toxicity of these reactive intermediates towards bone marrow cells has also been suggested (*9*).

In recent studies, cationic nitrogen centered radical intermediates have been detected during myeloperoxidase and hydrogen peroxide mediated metabolism of aryl amines (*11, 26*). Further, as discussed previously, some aromatic amines during their peroxidase metabolism have been detected to produce phenyl radicals (*11, 25, 26*). In aminoglutethimide treated promyelocytic leukemia cells (HL-60), protein radical formation has been detected which is suggested to be produced due to reactivity of phenyl radicals with cellular protein. Interestingly, the molecular weight of protein radicals detected is in the range of metabolizing enzyme myeloperoxidase itself. The formation of drug induced protein radical formation during peroxidase metabolism of aromatic amines has been suggested as a mechanism of toxicity for drug induced agranulocytosis (Figure 1.7) (*25*). Further, N-centered cationic radicals are known to cooxidise glutathione, NADH and ascorbate during myeloperoxidase mediated metabolism

of aryl amines (*1, 27-29*). These cationic free radicals are also known to form DNA adducts (*1*). However, these findings have not been associated with drug induced agranulocytosis.

1.4 Free radical metabolites of aromatic amines and their intracellular targets

Toxicities of many xenobiotics including aromatic amines are associated with generation of free radical intermediates during their enzymatic metabolism. Free radicals are the species (atoms or molecules) which contain one unpaired electron (*30*). Due to presence of this unpaired electron, free radical species are extremely unstable and reactive. These species in order assuage their instability react with their surrounding molecules which they do so either by abstracting an electron or hydrogen atom from a molecule, adding across an unsaturated bond, or by forming radical-radical dimer (*31*).The former reaction lead to extensive cellular damage. Free radical mediated oxidative damage is also suggested to play a potent role in aging related health problems including cancer and heart disease (*32*). These free radicals including oxygen derived, radicals are capable of interacting with the biomolecules of cells, subsequently resulting in their damage. Moreover, radical initiated processes are deleterious because they are propagative, i.e., radical interaction with cell components may produce secondary and tertiary free radicals derived from lipids, amino acids, glutathione, ascorbic acid, or components of nucleic acids. Free radicals, once generated, may react with molecular oxygen to produce univalent reduced superoxide anion ($O_2^{\cdot -}$) and

further to a peroxyl radical. Cumulatively these cascade reactions may result in death of cells (*33*). It can be realized from our previous discussions that aromatic amines are also involved with the generation of these highly reactive free radical intermediates during their enzymatic metabolism and how they may react with intracellular antioxidants like glutathione, ascorbate, NADP or biomolecules like protein and nucleic acids (*1*, *25-29*).

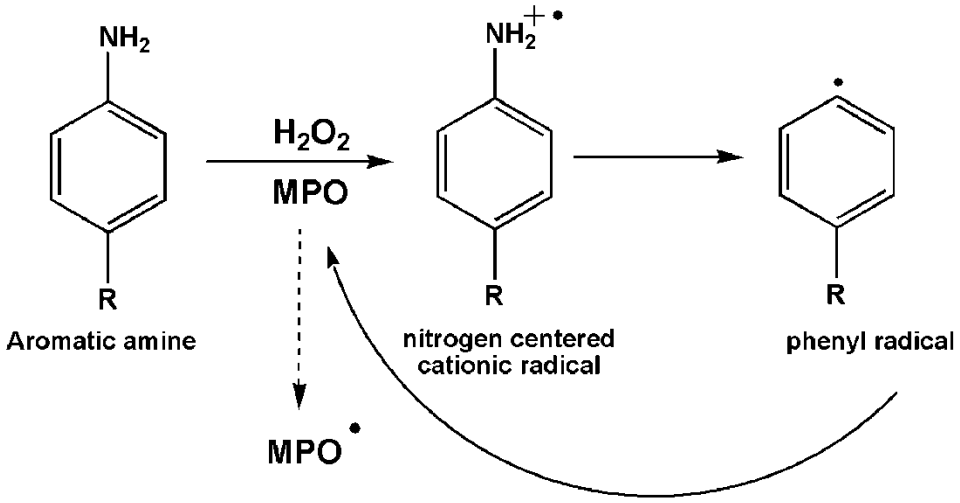


Figure 1.7 Phenyl radical induced protein radical formation

Representation of proposed mechanism for drug induced protein radical formation during peroxidase mediated generation of phenyl radical production in agranulocytosis by Siraki *et. al*. 2007.

1.4.1 Free radical induced oxidation of glutathione

Glutathione is a natural antioxidant found in cells. It is a major non-protein thiol present in all organs and virtually in all mammalian cells. It is a tripeptide composed of three sub-units glutamic acid, cysteine and glycine and contains the active group represented as a thiol (-SH), which is part of the cysteine residue. Glutathione is present in both free forms, and protein bound. The latter is found in the form of glutathionylated proteins. It is present in reduced form mostly which can be converted to oxidized formed in presence of oxidizing species like free radical. In a normal cell, glutathione is present at about 1 to 10 mM for in which the reduced form is predominating over the oxidized form. The oxidized form can be converted back to reduced form by the action of the enzyme glutathione reductase, present in the cells. Glutathione act as a major anti-oxidant and helps in protecting cells against oxidative stress induced to reactive oxygen species and free radicals. It also helps in detoxification process where it covalently interacts with xenobiotics to aid excretion as mercapturic acids in the urine and feces (*34*).

1.4.2 Free radical induced lipid peroxidation

The plasma membrane is composed of phospholipids which are abundant in poly unsaturated fatty acids (PUFA). Due to the presence of unsaturated bonds and allylic hydrogens these PUFAs of phospholipid membranes are highly susceptible to oxidation by free radicals (*33*). These free radicals may be generated during regular metabolic processes where they are generally scavenged

by other anti-oxidants like glutathione, ascorbate or α-tocopherol etc, preferably. However, when the flux of free radicals or reactive oxygen species increases, as during drug metabolism, it may result in depletion of protective anti-oxidants in the cells, and ultimately may result in radicals reacting with double bonds of PUFAs of plasma membrane. This results in lipid peroxidation and can cause lethal insult to cell integrity leading to hampered fluidity of membrane and damage of cellular compartmentalization (*33*).

Chapter 2
Rational[1]

As discussed in the previous chapter, free radical generation may result in damage to cellular biomolecules like lipids, proteins and DNA. Although xenobiotics may induce the generation of reactive oxygen or reactive nitrogen species that react with cellular macromolecules, reactions may occur directly by xenobiotic free radical metabolites themselves.

O'Brien's group has performed a series of studies that explored the relative reactivity of different xenobiotic and drug metabolites produced by horseradish peroxidase (HRP)/H_2O_2. HRP is a peroxidase enzyme having similar activity as that of human MPO, which can be used as a substitute of MPO. These reactions were monitored by detecting the oxygen consumption that occurred once these metabolites reacted with GSH, NADH, or arachidonate (*27, 35, 36*) through the following reactions:

$$\text{R-NH}_2 \xrightarrow{\text{HRP/H}_2\text{O}_2} \text{R-NH}_2^{\bullet+}$$

$$\text{R-NH}_2^{\bullet+} + \text{GSH} \xrightarrow{-\text{H}^{\cdot}} \text{R-NH}_2 + \text{GS}^{\bullet} \xrightarrow{\text{GS}^-} \text{GSSG}^{\bullet-} + \text{O}_2 \rightarrow \text{GSSG} + \text{O}_2^{\bullet-}$$

$$\text{R-NH}_2^{\bullet+} + \text{NADH} \xrightarrow{-\text{H}^{\cdot}} \text{R-NH}_2 + \text{NAD}^{\bullet} \xrightarrow{\text{O}_2} \text{NAD}^{+} + \text{O}_2^{\bullet-}$$

$$\text{R-NH}_2^{\bullet+} + \text{arachidonate} \xrightarrow{-\text{H}^{\cdot}} \text{R-NH}_2 + \text{arachidonate}^{\bullet} \xrightarrow{\text{O}_2} \text{arachidonate-OO}^{\bullet}$$

[1] *A version of this chapter has been published. Narwaley,M et.al. 2011.Chemical Research Toxicology.24(7).1031-1039.*

These reactions assume that the oxidizing species is the nitrogen-centered cation radical ($R\text{-}NH_2^{\bullet+}$), which is the first product that is formed in these reactions.

Apart from reactivity of arachidonate with free radical (*35*), it has been shown by Yu, 2001, that PUFAs like linoleic acid have the properties to scavenge carbon centered free radicals (*37*). Further, a differential reactivity of the isomers of conjugated linoleic acid towards scavenging properties for carbon radicals has also been demonstrated (*32*).

Recently, in the studies performed by Siraki *et. al.* 2010, phenyl radicals have been detected during peroxidase mediated metabolism of aryl amine xenobiotics like aminoglutethimide, procainamide and congeners in the presence of hydrogen peroxide. Further, as discussed in the previous chapter, these metabolites were implicated in the generation of protein radicals which appeared to form predominantly on myeloperoxidase (MPO) in HL-60 cells (*25*). Further, as discussed in previous chapter, that presence of unsaturated bonds on lipids make them prone to carbon centered free radical target which leads to lipid peroxidation **(*33*)**. This suggests a possibility that PUFAs can also act as targets of phenyl radicals.

However, it is not clear why certain peroxidase substrates show poor reactivity towards GSH or NADH while they are suggested to produce the same N-cationic radical as shown by Subrahmanyam, *et. al.*, 1987. Moreover, it is not known if the phenyl radical generated in these types of peroxidase reactions will show the same pattern of reactivity. It is possible that different reactive metabolites (*viz.* N-cationic radical and phenyl radical) may have different

reactivity towards different biomolecules. It is important to study the reactivity of the phenyl radical metabolites of these drugs since they may play a role in their propensity to induce agranulocytosis (neutrophil depletion). Aside from protein, there may be other significant intracellular targets for drug-derived phenyl radical metabolites.

2.1 Hypothesis

1. We hypothesize that the aromatic amine xenobiotics on peroxidase/H_2O_2 mediated metabolism will generate free radical reactive metabolites which will react with different cellular targets such as glutathione and PUFAs. A schematic representation of hypothesis is shown in Figure 2.1 and Figure 2.2.

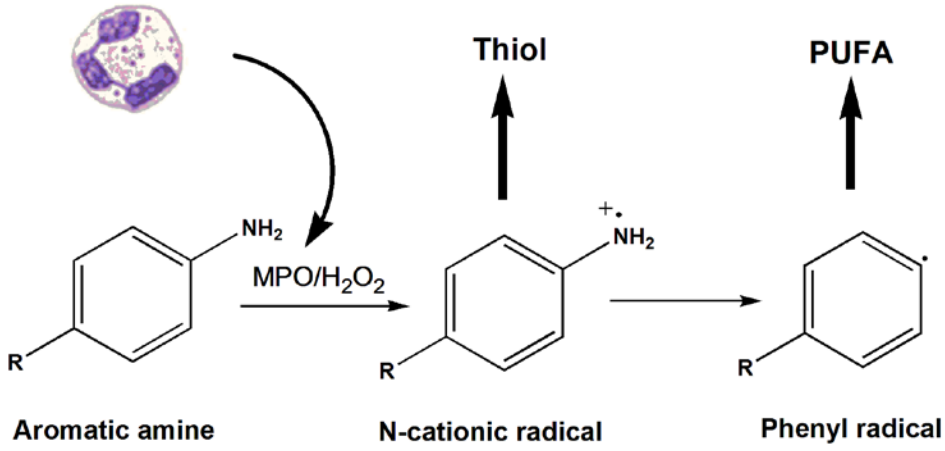


Figure 2.1 Thiol and PUFA as target molecules of aromatic amine reactive metabolites

Peroxidase mediated metabolism of aromatic amine resulting in formation of N-cationic radical followed by phenyl radical formation. N-cationic radical intermediate will react with thiols as targets while for phenyl radical metabolite will react with PUFA.

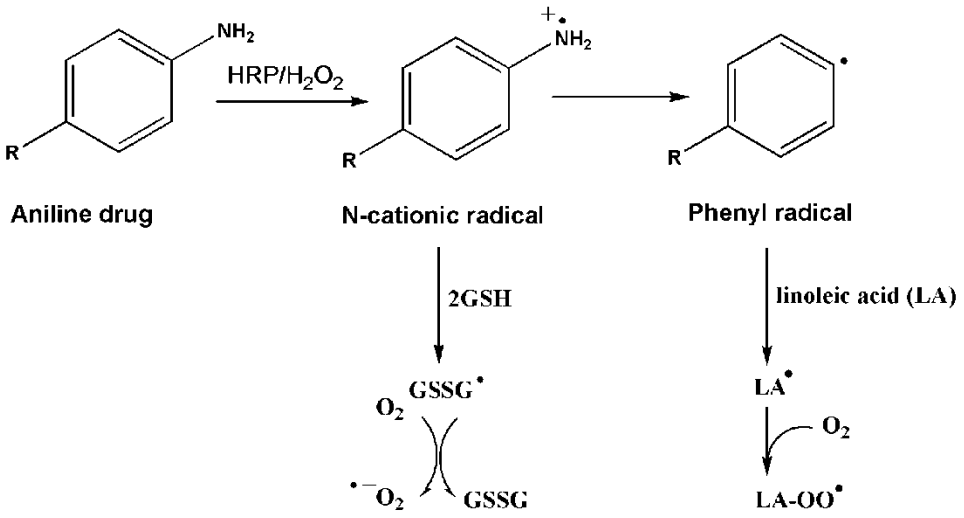


Figure 2.2 Hypothesis-Reaction of free radical intermediates of aromatic amine with different targets during peroxidase metabolism

Suggested reaction mechanism for N-cationic radical metabolite towards glutathione and phenyl radical towards PUFA linoleic acid (LA).

2. We suggest that aromatic amines which do not generate detectable amount of phenyl radicals should have differential reactivity towards glutathione. Further, those aromatic amine congeners which generate phenyl radical on peroxidase/H_2O_2 will react with poly unsaturated fatty acids like linoleic acid, docosahexaenoic acid, sorbic acid and acrylic acid and will cause per-oxidation at unsaturated bonds of PUFAs (Figure 2.3).

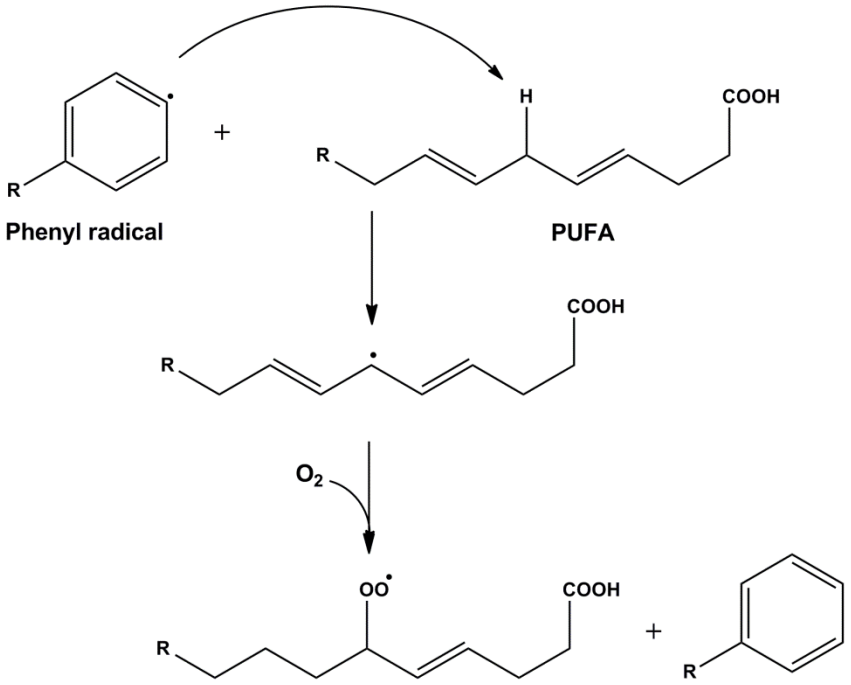


Figure 2.3 Phenyl radical induced lipid peroxidation reaction

Proposed reaction mechanism for phenyl radical interaction with unsaturated bond of PUFA leading to lipid peroxide formation.

2.2 Objectives

To test our hypothesis, we had planned our objectives as follows:

1. To detect the phenyl radical generated during peroxidase/H_2O_2 metabolism of aromatic amines and to test its reactivity towards different cellular targets.
2. To differentiate the reactivity pattern with different intracellular target molecules between N-centered cationic radical and phenyl radical generated during peroxidase/H_2O_2 metabolism from aromatic amines and its congeners.
3. To test if carbon centered phenyl radical reacts with unsaturation site of PUFA and cause lipid peroxidation. Further, we would test to know the effect of phenyl radical metabolite interaction with PUFAs on aromatic amine induced protein radical formation.

Chapter 3
Materials and Methods[2]

3.1 Reagents

Drugs and related chemicals, horseradish peroxidase type VI (HRP, EC: 1.11.1.7), sodium phosphate, potassium dichromate, 1,1,3,3-tetramethoxypropane, 2,4-dinitrophenyl hydrazine (DNPH), glutathione, linoleic acid, sorbic acid, docosahexaenoic acid, hexanoic acid, propanoic acid, acrylic acid, cold fish gelatin were purchased from Sigma-Aldrich (Oakville, ON). AG was purchased from Toronto Research Chemicals (Toronto, ON). Hydrochloric acid, Acetonitrile and HPLC grade water was purchased from Caledon laboratory chemicals (Georgetown, ON). 0.1 M sodium phosphate buffer pH 7.4 (PB) was prepared and used after metal chelation by incubating overnight with Chelex-100 resin (Bio-Rad Laboratories, Mississauga, ON), and was used (referred to as PB buffer). DMPO was purchased from Cedarlane Laboratories (manufactured by Dojindo Molecular Technologies, Inc., Japan) and was used without further purification. 2-Methyl-2-nitrosopropane (MNP) was purchased from Sigma-Aldrich and was prepared the day before experimentation by dissolving a solution of 2 mg/ml in buffer by mixing overnight in darkness at 32 °C. The different aromatic amine xenobiotics and congeners used are shown in Table 3.1. Table 3.2 shows different unsaturated and saturated fatty acids used this study.

[2] *A version of this chapter has been published. Narwaley,M et. al. 2011.Chemical Research Toxicology.24(7).1031-1039.*

Table 3.1 Structures of aromatic amines and congeners used during study.

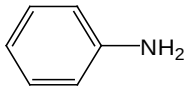
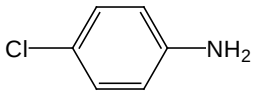
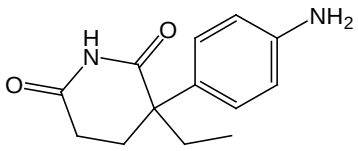
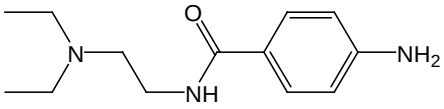
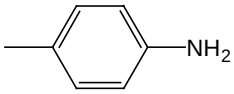
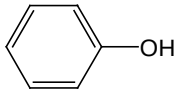
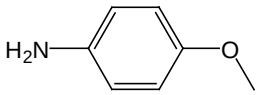
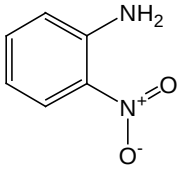
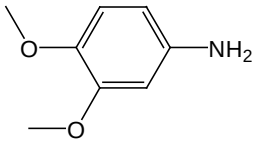
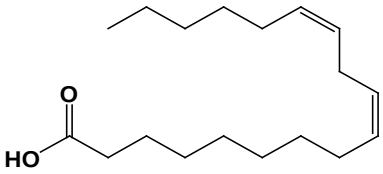
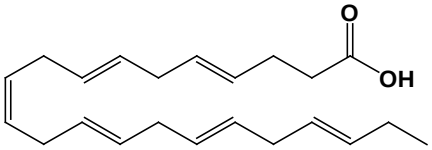
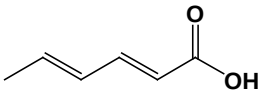
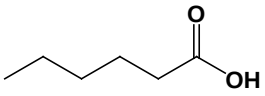
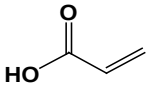
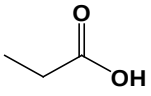
Xenobiotic	Chemical Structure
Aniline	
4-chloroanilne	
Aminoglutethimide	
Procainamide	
p-toluidine	
Phenol	
p-aniside	
2-nitroanilne	
3,4-dimethoxyanline	

Table 3.2 Structures of unsaturated and saturated fatty acids used during study

Fatty acid	Structure
Linoleic acid	
Docosahexaenoic acid	
Sorbic acid	
Hexanoic acid	
Acrylic acid	
Propionic acid	

3.2 Experimental methods:

3.2.1 Oxygen consumption analysis:

3.2.1.1 Principle:

To test if there is any oxidation-reduction reaction is occurring between aromatic amines drugs and selected targets in the presence of horseradish peroxidase and hydrogen peroxide, we used Clark type oxygen electrode. Clark electrodes work on the principle in which it consist of sensing platinum cathode and an anode of silver. At 800 mV of polarizing voltage oxygen can be reduced into hydroxide ions which results in generation of current which is proportional to the oxygen concentration in the sample (*38*). The electrode is covered with a cellophane membrane which is permeable to oxygen (*39*). Under the permeable membrane, the electrode is immersed in an electrolyte which helps in carrying the current. The current measured is read by an oxygen monitor which represents the current recorded into the form of oxygen content. The electrode reactions can be presented as follows (*38*, *40*).

At anode (Ag) with electrolyte KCl or KBr:

$$2Ag + 2Cl^- \rightarrow 2AgCl + 2e^-$$

At cathode (Pt):

$$\frac{1}{2}O_2 + 2e^- + H_2O \rightarrow 2OH^-$$

Net Reaction:

$$2Ag + 2Cl^- + \frac{1}{2}O_2 + 2e^- + H_2O \rightarrow 2AgCl + 2e^- + 2OH^-$$

During oxidative reactions, free radical species may reduce the oxygen molecule present in the sample which appears as depletion in oxygen content in oxygen monitor. This oxygen uptake recorded with the help of Clark electrode is often indicative of free radical mediated reactions (*27, 29, 35*).

3.2.1.2 Method:

Oxygen consumption was recorded using a YSI 5300 biological oxygen monitor (Yellow Springs Inc., Yellow springs, OH) with a Clark oxygen electrode. The data was recorded using a DATAQ interface (Akron, Ohio, USA) which allowed for a windows-based PC to record the oxygen consumption graph. Rates of oxygen consumption were calculated after the data was imported into MS Excel.

For reactions measuring oxygen consumption in reactions containing GSH, 400 µM GSH, 1600 µM drug and 50 µM H_2O_2 were added to PB buffer, and the reaction was initiated with the addition of 0.01 µM HRP. For reactions containing LA (LA), 50 mM LA, 5 mM drug, and 1 mM H_2O_2, were added to PB buffer and the reaction was initiated with the addition of 1µM of HRP. Similar reactions conditions were followed for testing procainamide and its congeners in the presence of LA, HRP and peroxide. Other unsaturated aliphatic acids were used with the concentrations indicated in the results section and the reactions were performed similar as discussed above. The schematic representation of method is shown in Figure 3.1.

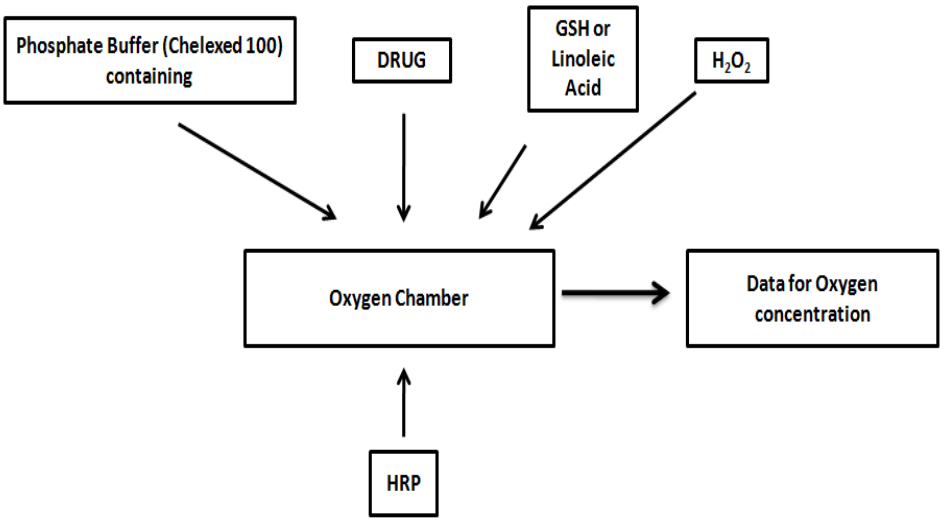


Figure 3.1 Scheme of oxygen electrode method

Reactants were added in the oxygen chamber fitted with Clark electrode and containing phosphate buffer 7.4pH (chelax100). The reaction was initiated with addition of HRP. The change in oxygen concentration was recorded with the help of oxygen monitor.

3.2.2 Electron spin resonance (ESR) spectrometry:

3.2.2.1 Principle:

Electron spin resonance (ESR) also called as electron paramagnetic resonance is a technique to detect paramagnetic species like free radicals which are deficient in one electron**.** The basic principle of ESR is very similar to that of nuclear magnetic resonance (NMR). Like NMR, it is also based on the interaction of magnetic moment of sub-atomic particles (in this case electrons) with the electromagnetic field applied. The magnetic moment of an electron arises due to its "spin" under the influence of external magnetic field applied. Under the influence of magnetic field, the electron aligns its spin either parallel or anti-

parallel to the direction of magnetic field applied (*41*). This alignment of spin brings the electron spin in resonance with the magnetic field and leads to an upper (+) and lower (-) energy state of ±1/2. The difference of these two energy states is proportional to the strength magnetic field applied (*41, 42*).

$$\Delta E = g\mu B \qquad (1)$$

Where, ΔE is the difference in two energy levels of spin, μ is magnetic moment, g is g-factor and B is magnetic field. Similar to NMR, the interaction of electron spin with the neighboring nuclei produces splitting in the spectrum.

Free radicals produced in low yields and high instability is one of the limitations of ESR studies which makes difficult to detect them directly. To overcome this problem, the spin trapping technique has been introduced. A spin trap reagent is a diamagnetic species which interacts with the free radical under observation to produce a new stable free radical adduct detectable by ESR. These spin traps can be identified based on their splitting constants. The spin traps used most often are nitroso and nitrone species (Figure 3.2) (*43*). The reacting radical interacts with the nitrogen atom of a nitroso spin traps while it interacts with unsaturated carbon in case of nitrone spin traps (*43*). A commonly used nitroso compound is 2-methyl-2-nitrosopropane (MNP) which we have used in our studies. Nitrone spin traps commonly used compounds are phenyl-N-*tert*-butylnitrone (PBN), and nitrone spin trap used is 5,5-dimethyl pyrroline-N-oxide (DMPO) (*25, 43*).

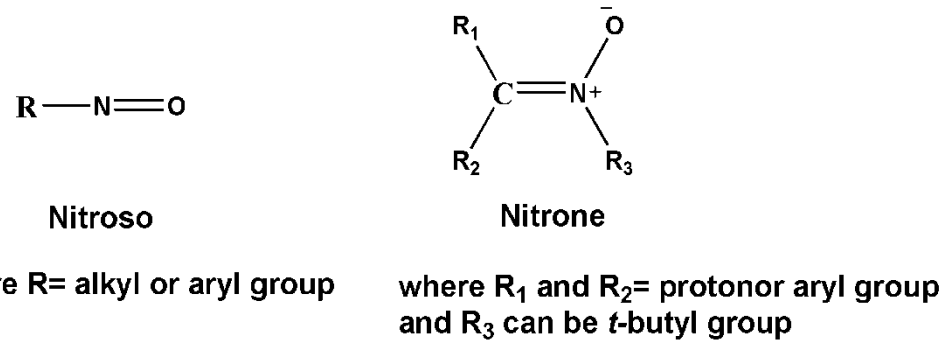


Figure 3.2 structure of nitroso and nitrone spin traps.

In our studies, a phenyl radical was produced during peroxidase metabolism of aromatic amines in presence of hydrogen peroxide (*11*) which was spin trapped with the help of nitroso compound MNP. The phenyl radical formed following the formation of N-cation radical reacts with MNP nitroso spin trap to form a new stable MNP-phenyl radical adduct which is detectable by ESR as shown in Figure 3.3.

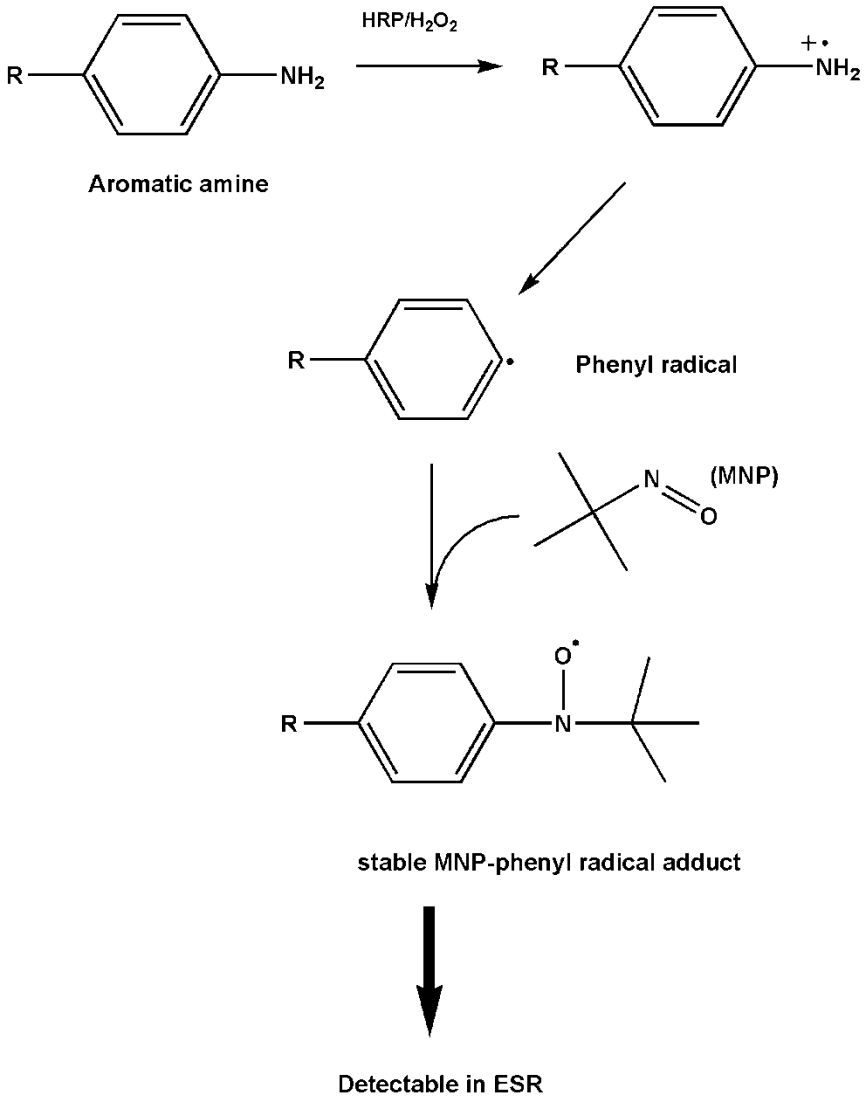


Figure 3.3 Spin trapping of phenyl radical by nitroso

Peroxidase/H_2O_2 mediated formation of phenyl radical from aromatic amine interacts with MNP to from stable nitroso radical adduct (MNP-phenyl radical) which is detectable in ESR.

3.2.2.2 Method

Experiments were designed to test the effect of LA on the production of phenyl radical formation from AG. We used an Elexsys E500 EPR spectrometer

to detect the formation of phenyl radical from aromatic amine drug with the following instrument parameters: frequency = 9.79 GHz, modulation = 100 kHz, modulation amplitude = 0.4 G, power = 20 mW, gain = 60 dB, scan time = 163 s, time constant = 163 ms. The phenyl radical was detected first by using MNP spin trap. Once, the radical formation from aminoglutethimide in presence of HRP and hydrogen peroxide in phosphate buffer was confirmed the reaction was carried out in PB buffer, and contained 0 - 100 mM LA, 22 mM MNP, 5 mM AG, 1 mM H_2O_2, and 0.1 µM HRP (added last). The reaction was briefly vortexed and transferred to a flat cell for data acquisition. After the data was obtained, we performed simulations of the spectra using WinSim (public EPR software tools, NIEHS/NIH).

3.2.3 Determination of MDA from HPLC method:

3.2.3.1 Principle:

Lipid peroxidation leads to formation of aldehydes and ketones. Amongst these aldehydes, malondialdehyde (MDA) is the most frequently formed and used as a marker for lipid oxidation reactions. Formation of malondialdehyde was confirmed by HPLC.

We modified a previously reported HPLC method for the determination of MDA to serve our purpose in the biochemical system (*44*). Pre-column derivatization of MDA using 2,4-dinitrophenylhydrazine (DNPH) was performed prior to injection into the chromatograph which was later confirmed by mass

spectrometry (Figure 3.4) (*45*). A standard of MDA was prepared from its stable precursor 1,1,3,3-tetramethoxypropane (TMP) which was derivatised using DNPH. A positive control of MDA derived from sorbic acid was also prepared and confirmed.

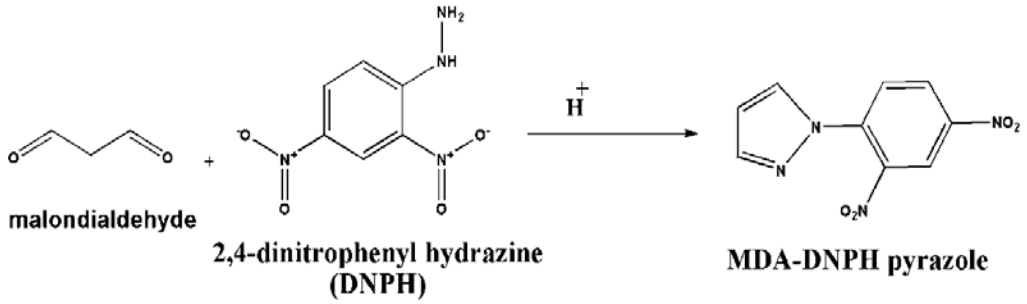


Figure 3.4 Derivatization of MDA with DNPH

Derivatization of malondialdehyde with DNPH to produce pyrazole derivative which is detectable in HPLC.

3.2.3.2 Method

(a) MDA Standard preparation:

MDA standard was prepared by following the method of Trivella *et al.* (*46*). In brief, stock solutions of MDA were prepared by dissolving TMP (938 µl) in 1 M HCl (500 µl), and incubating the solution for 15 min at room temperature (RT). Afterwards, the solution was neutralized to pH 7 using 1 M NaOH to avoid the possible polymerization of MDA in acidic pH (*46*). The solution was stable when kept at 2-8 °C for at least 1 month. Working standards of MDA were then obtained by proper dilutions in water.

(b) Derivatization procedure:

A stock solution of 50 mM DNPH was prepared in acetonitrile:HCl (3:1) (*47*). An aliquot of 120 µl of 5 mM of MDA working standard was added with 80 µl stock DNPH solution to give a final concentration of 20 mM in final mixture. The reaction mixture was heated at 50 °C for 5 min (*48*), and then it was allowed to cool at room temperature. The MDA-DNPH derivative was extracted with 2 volumes of methyl-*t*-butyl ether by mixing at 1000 rpm for 5 min at RT. The upper ether layer was separated carefully and the solvent was evaporated under a gentle stream of nitrogen. The residue was dissolved in mobile phase described below and directly injected into the chromatograph.

(c) Determination of MDA-DNPH:

MDA-DNPH was chromatographed on a Biobasic-18 RP column (Thermo Scientific) (250 mm×4.6 mm) using an isocratic mobile phase composed of acetonitrile : water at 0.2% acetic acid (35 : 65) and pumped at a flow rate of 1 ml/min. The mobile phase was filtered and degassed prior to running in HPLC. The liquid liquid extract was diluted 100 times with the eluent, and 50µl was injected into the HPLC with flow rate of 1 ml/min. The column eluent was monitored at 310nm using a UV detector (*44*). The MDA-DNPH peak was identified by peak fortification at t_R 10.5 min. A blank chromatogram was obtained by extracting a blank sample treated similarly. To further confirm the identity of the derivative, the peak volume at t_R 10.5 min was collected and the MDA-DNPH complex was identified by mass spectrometry.

(d) Sorbic acid-derived MDA by chemical peroxidation:

Sorbic acid was oxidized under mild conditions with 0.1 N potassium dichromate and 0.1 N sulfuric acid, and peroxidation products were monitored (*49, 50*). Sorbic acid (500 µl of 1 mg/ml solution) was treated with 500 µl of 0.1 N potassium dichromate and 500 µl of 0.1 N sulfuric acid. The reaction was heated at 99°C for 5 min (*51, 52*). The reaction mixture was allowed to cool at RT and DNPH was added in an aliquot to have final concentration of 20 mM, then the derivatization and extraction procedures were followed as described above. Finally, the residue after extraction was diluted in the mobile phase and injected into the HPLC under the same conditions previously adopted. The peak of MDA, a lipid peroxidation product of the above reaction, was identified by t_R matching with the standard.

(e) Determination of sorbic acid-derived MDA -through oxidation by enzymatically generated free radicals:

Aminoglutethimide (5 mM) and sorbic acid (20 mM) were allowed to react in presence of H_2O_2 (1 mM), and the reaction was initiated by adding HRP (1 µM) in 0.1 M phosphate buffer pH 7.4 (Chelexe 100 treated). The reaction was incubated for 30 min, and then it was terminated by the addition of catalase (20 nM). The reaction mixture was treated with DNPH as described under “derivatization procedure”. The DNPH-treated mixture was extracted and chromatographed as previously described. All necessary controls were also prepared and likewise processed and analyzed by HPLC. A peak at 10.5 min was

observed and was accordingly identified as the MDA-DNPH derivative on the basis of retention time matching with the MDA standard and the sorbic acid positive control reaction describe above. As a confirmatory test, the biochemical sample was co-chromatographed with MDA standard, and peak response enhancement was perceived. None of the controls gave any peaks at the t_R of MDA-DNPH.

3.2.4 Western blot analysis with HL-60 cell lysate by immunospin trap

3.2.4.1 Principle

It has been discussed previously that how phenyl radicals are involved in formation of protein radicals (*25, 26*). The protein radical thus formed can be trapped by using 5,5-dimethyl pyrroline-N-oxide (DMPO) to form stable protein-DMPO radical adduct. This protein-DMPO radical adduct is detected by using an anti-DMPO antibody by performing immunoassay like ELISA or western blotting (Figure 3.5) (*25*).

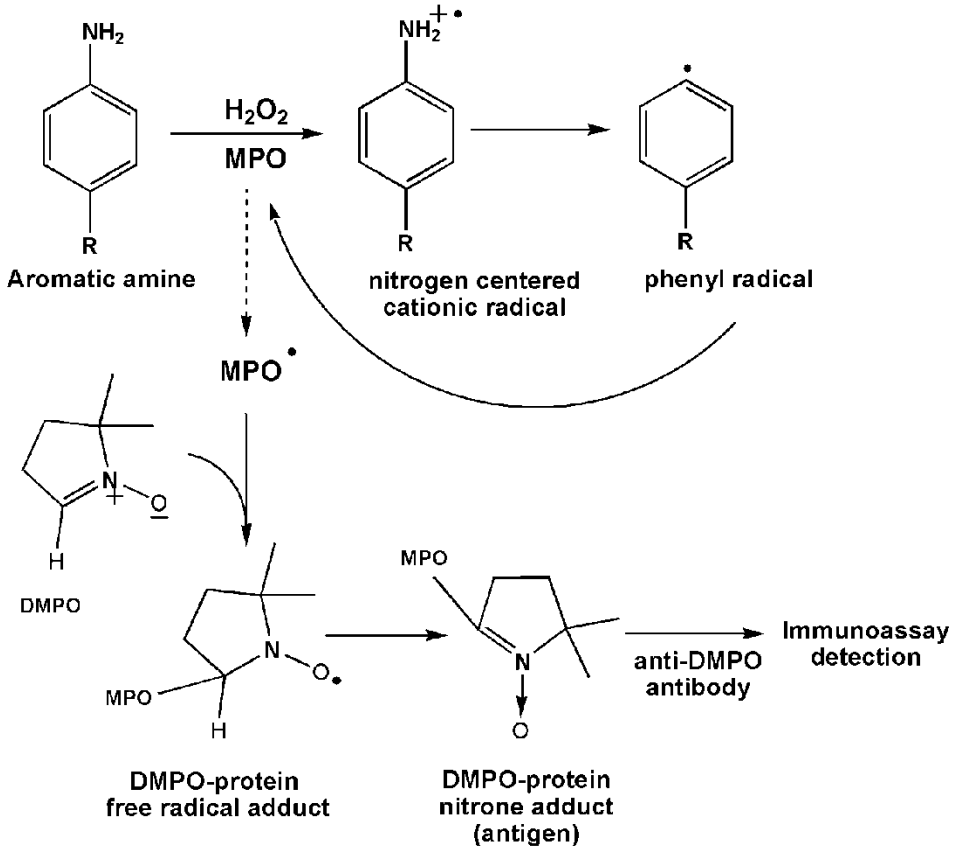


Figure 3.5 Proposed mechanism of immunospin trapping of protein radical

Proposed mechanism of immunospin trapping using DMPO, of aromatic amine induced protein radical formation and its immunoassay detection.

3.2.4.2 Method

We tested the effect of presence of unsaturated fatty acids on the phenyl radical induced protein radical formation in the cells. For that we planned a western blot experiment.

HL-60 cells were bought from ATCC and grown in RPMI media containing 10% serum. Prior to treatment HL-60 cells (2 x 10^6 cells/ml) were lysed by using RIPA buffer (0.05 g sodium deoxycholate, 100 µL of Triton X-

100, and 10 µL of 10% SDS in 10 ml of PBS). 100 µL of cell lysate was treated with DMPO (100 mM), AG (5 mM), DHA (0.5, 2, 10, and 25 mM) or LA (50 mM) in different combinations. Here we tested a dose dependent effect of unsaturated fatty acid over the AG induced protein radical formation. The reaction was initiated by adding glucose (5 mM) and GO (5 mU/100 µL) for generating H_2O_2. Controls were performed in absence of each reactant. The reaction was incubated for 4 hrs at 25º C. The treated cell lysate and controls were reduced by using 10% TCEP solution (Thermo Scientific) and heat treated for 5 min at 95ºC with constant shaking at 500 rpm. Reduced samples were then loaded in a 1.0 mm 10% polyacrylamide gel. The proteins were transferred from polyacrylamide gel over the nitrocellulose membrane using a semi-dry transfer module (Bio-Rad Laboratories). The membrane was blocked overnight at 4ºC using 4% cold fish gelatin or bovine serum albumin (BSA) and then was treated with anti-DMPO antibody (1:5000, Oxford Life Sciences) to detect DMPO-protein adducts. After 1 h, the membrane was washed (4×) and treated with secondary antibody (goat anti-rabbit HRP-conjugated, Thermo Scientific) for 1 h. After washing (4×) the membrane was treated with chemiluminescence HRP substrate reagent (Immobilon Western^{TM}, Millipore) for 2 min and exposed to CL-XPosure^{TM} clear blue X-ray film (Thermo scientific). The same membrane was then reprobed for MPO and GAPDH using anti-MPO and anti-GAPDH (loading control) (1:5000 of each) after stripping. The nitrocellulose membrane was stripped by treating the membrane with stripping buffer (1.5 g/L glycine, 0.1 g/L SDS, 1 ml/L Tween-20, in water adjusted to pH 2.2) for 20 min at room temperature. The membrane was

washed twice with PBS for 10 min, and then twice with TBS-Tween for 5 min (30 min wash overall).

Chapter 4
Results and Discussion[3]

4.1 Free radicals metabolites of aromatic amines catalyzed oxygen consumption with either GSH or LA:

There was a differential response found between certain aromatic amine substrates in their selectivity for the co-oxidation of either GSH or LA. As shown in Figure 4.1, the co-oxidation of AG with LA by HRP/H_2O_2 resulted in significant oxygen consumption. However, no oxygen consumption was detected if LA was replaced with GSH. On the other hand, p-anisidine was effective in co-oxidizing GSH but not LA. We further investigated this differential reactivity using other aromatic amine substrates (Table 4.1). We found that p-toluidine, p-anisidine, and 3,4-dimethoxyaniline all induced significant oxygen consumption in the presence of GSH and peroxidase/H_2O_2. Phenol, which is known to catalyze oxygen consumption under these conditions, was used as a positive control (*27*). Interestingly, these compounds did not induce significant oxygen consumption when LA was used instead of GSH (with the exception of p-toluidine). Aniline, 4-chloroaniline, and AG induced significant oxygen consumption in the presence of LA and HRP/H_2O_2. Interestingly, there appeared to be a correlation between drugs that have been shown to form a phenyl radical (*11*) and drugs that catalyzed oxygen consumption with LA. 4-Methylaniline (p-toluidine) displayed significant oxygen uptake with both GSH and LA, suggesting that it forms both R-$NH_2^{\bullet+}$ and

[3] *A version of this chapter has been published. Narwaley,M et.al. 2011.Chemical Research in Toxicology.24(7).1031-1039.*

phenyl radicals in similar proportions. It was previously shown by O'Brien that p-phenetidine induced significant oxidation of arachidonate and NADH (*53*); it is possible that p-toluidine has similar reactivity. However, there were two compounds that did not correlate. 2-Nitroaniline did not induced oxygen consumption with GSH and very low oxygen uptake with LA, which was consistent with the latter since a phenyl radical was not detected (*Siraki et.al, 2010*). It was surprising; however, that procainamide did not induce oxygen consumption in either case even though a phenyl radical has been reported in a MPO/H_2O_2 system (*26*).

In order to determine why procainamide did not induce oxygen consumption with LA, we compared the activity of different procainamide congeners in this system (Table 4.2).We found that different substitutions at the amide portion of the molecule resulted in different rates of oxygen consumption with LA in the following order: procainamide < procaine < p-aminobenzamide < benzocaine < *p*-aminobenzoic acid. Seemingly, the triethylamine and amide substructures resulted in attenuated oxygen consumption in the presence of LA. The ester or carboxyl containing analogs (benzocaine, *p*-aminobenzoic acid) were more efficient in catalyzing oxygen consumption in the presence of LA.

In order to investigate the effect of the unsaturated bonds in LA, we compared the reactivity of different unsaturated fatty/alkyl acids (Table 4.3). In this set of fatty/alkyl acids, we found that sorbic acid (2,4-hexadienoic acid) catalysed the greatest extent of oxygen consumption in the presence of 4-

chloroaniline and HRP/H_2O_2. On the other hand, the oxygen consumption rate with hexanoic acid (the saturated congener of sorbic acid) was approximately 40-fold lower. This suggested that the conjugated double bonds in sorbic acid played an essential role in oxygen consumption. The relative order of effectiveness in catalyzing oxygen consumption was sorbic acid >> DHA > acrylic acid > hexanoic acid > propionic acid. There was a direct relationship between the number of double bonds and oxygen consumption with the exception of sorbic acid. The latter is an α,β-unsaturated compound and its conjugated bonds may explain its enhanced reactivity.

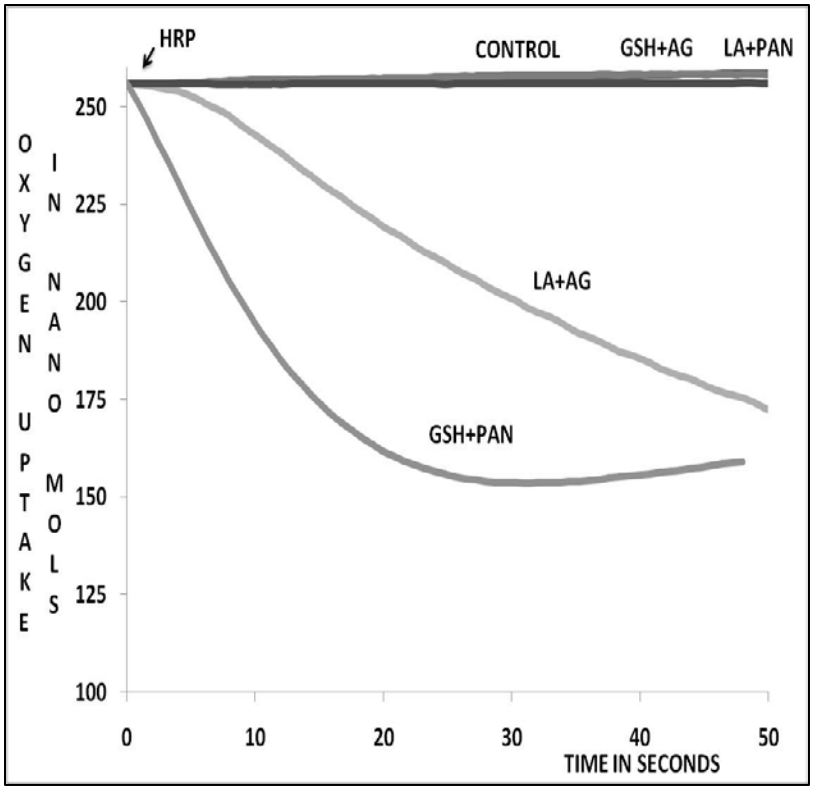


Figure 4.1 Oxygen uptake shown by GSH and LA in presence of aromatic amines

Representation of oxygen uptake by GSH (1600 µM) and LA (50 mM) in presence of AG, H_2O_2 and HRP showing total oxygen uptake in nmol with respect to time in seconds. 400 µM of AG, 50 µM of H_2O_2 and reaction was initiated by adding 0.01 µM of HRP with GSH while, 5 mM of AG, 1 mM of H_2O_2 was added with LA and the reaction was initiated by adding 1 µM of HRP. The reactions were carried at room temperature in phosphate buffer of pH7.4 (treated overnight with Chelax 100).

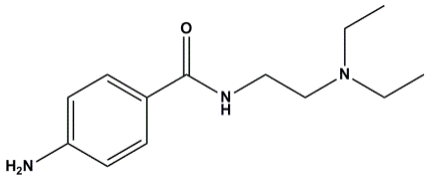
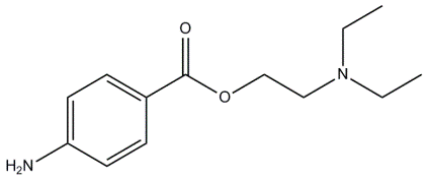
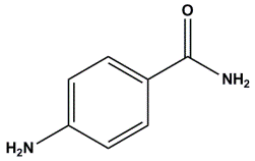
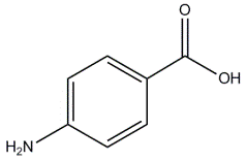
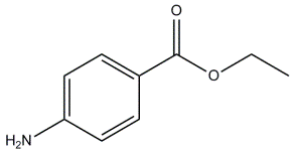
Abbreviations (above figure): PAN, *p*-anisidine; LA, linoleic acid; AG, aminoglutethimide; GSH, glutathione.

Table 4.1 Oxygen consumption of GSH and linoleic acid (LA) in HRP/H2O2 metabolism of aromatic amine drugs.

Substrate	Oxygen consumption (nmol/min)		Phenyl radical detected[†]
	GSH	LA	
none	0	0	
aniline	11±1	162±8	+
4-chloroaniline	30±2	228±11	+
aminoglutethimide	0	102±6	+
procainamide	0	0	+
p-toluidine	222±12	150±7	+
phenol	132±7	6±1	
p-anisidine	348±17	0	-
2-nitroaniline	0	8±1	-
3,4-dimethoxyaniline	156±7	10±1	-

Incubation conditions: The reaction was carried out in 0.1 M phosphate buffer containing. The reaction contained 400 µM substrate, 1600 µM GSH, 50 µM H_2O_2, and the reaction was then initiated with of 0.01 µM of HRP. In reactions containing LA, the same buffer was used to which we added 50 mM LA, 1mM H_2O_2, 5 mM of substrate, and was initiated with addition of 1 µM of HRP.
[†] from (*11*).

Table 4.2 Oxygen consumption of linoleic acid catalyzed by HRP/H2O2 metabolism of procainamide and related drugs

Substrate	Structures	LA Oxygen consumption (nmol/min)
procainamide		0
procaine		3±1
p-aminobenzamide		18±2
p-aminobenzoic acid		53±4
benzocaine		47±4

Incubation conditions: Reactions were carried out as described in Table 1 and in Materials and methods.

Table 4.3 HRP Mediated Oxidation of Selected Saturated and Unsaturated Alkyl/Fatty Acids by 4-Chloroaniline

Alkyl/fatty acid	Number of unsaturated bonds	Oxygen consumption (nmol/min)
Sorbic acid*	2	708±22
Hexanoic acid	0	17±2
Docosahexaenoic acid	6	401±12
Acrylic acid	1	19±1
Propionic acid	1	11±1**

Incubation conditions: Reactions were carried out as described in Table 1 (except that 5 mM 4-chloroaniline was used) and in Materials and Methods
*2,4-hexadienoic acid; 20 mM was used
** incubated with 20 mM 4-chloroaniline.

4.2 Effect of polyunsaturated fatty acids on phenyl radical detection by EPR:

In order to provide further evidence of a reaction between phenyl radical metabolites and PUFAs, we carried out EPR experiments. In a previous study, we assigned the spectrum derived from an MPO-catalyzed AG free radical metabolite to correspond with a carbon-centered phenyl radical with the following splitting constants: a^N = 14.4 G, $a^H_{m(3)} = a^H_{m(5)}$ = 0.93 G, and $a^H_{o(2)} = a^H_{o(6)}$ = 1.91 G (*25*). We repeated that experiment for comparison and found that a system containing MNP, AG, H_2O_2 and HRP produced a very similar spectrum with the following splitting constants: a^N = 14.5 G, $a^H_{m(3)} = a^H_{m(5)}$ = 0.93 G, and $a^H_{o(2)} = a^H_{o(6)}$ = 1.86 G (Figure 4.3A). The splitting constants were derived from a simulated spectrum of Figure 4.3A. In the presence of LA, this spectrum was attenuated (Figure 4.3B). This suggests that LA reacted with the phenyl radical that was produced from AG.

We performed further experiments with DHA since we showed in Table 4.3 that it was significantly effective in catalyzing oxygen consumption. As shown in Figure 4.3, DHA induced a dose-dependent decrease in the intensity of phenyl$^{\bullet}$/MNP spectrum. The signal was completely attenuated at 25 mM DHA (Figure 4.3E), although significant attenuation was observed with 2 mM DHA (Figure 4.3C). Interestingly, at the lowest concentration of DHA (0.5 mM, Figure 4.3B) we did not observe significant attenuation of the phenyl-MNP spectrum, but observed the formation of a new spectrum. Both LA and DHA have poor water solubility and the effect of solubility on modulating the EPR spectrum was

investigated with sorbic acid which was more soluble and demonstrated oxygen consumption. We found that sorbic acid was also able to attenuate the AG-derived phenyl radical spectrum, suggesting that solubility does not affect the ability of PUFAs to scavenge phenyl radicals (Figure 4.6). To investigate the relationship between oxygen consumption and the ability to attenuate phenyl radical detection, we performed dose-dependent oxygen consumption experiments using DHA. As shown in Figure 4.6, increasing concentrations of DHA resulted in increased oxygen consumption. Below 5 mM DHA it was difficult to determine oxygen consumption. Interestingly, we found with EPR experiments that 2 mM DHA was sufficient to attenuate the phenyl$^{\bullet}$/MNP spectrum by at least 50% (Figure 4.3C).

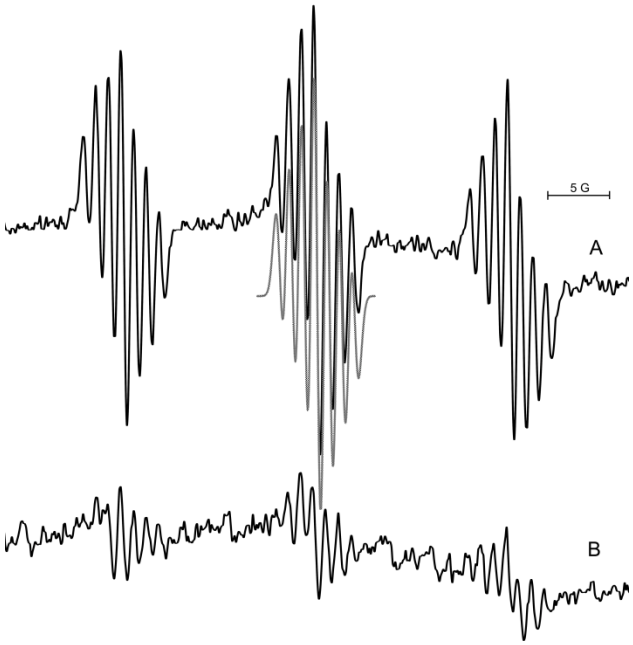


Figure 4.2 Attenuation of phenyl radical metabolite of aromatic amine by LA

ESR spin trapping experiments (with MNP) utilizing LA to attenuate AG free radical metabolite detection. The spectrum shown in (A) was repeated from a previous study in order to validate the findings from Figure 4.1. The dashed lines represent a simulation to the experimental spectrum. The correlation was r=0.997. The reaction was prepared and transferred to capillary tubes for recording. The ESR spectra resulting from reactions in PB containing 5 mM AG, 22 mM MNP, 1 µM HRP, 1 mM H_2O_2 with 0 mM LA (A) and 100 mM LA (B) are shown. The hyperfine splitting constants obtained from (A) were a^N = 14.5 G, a^H_m = 0.93 G, and a^H_o = 1.86 G.

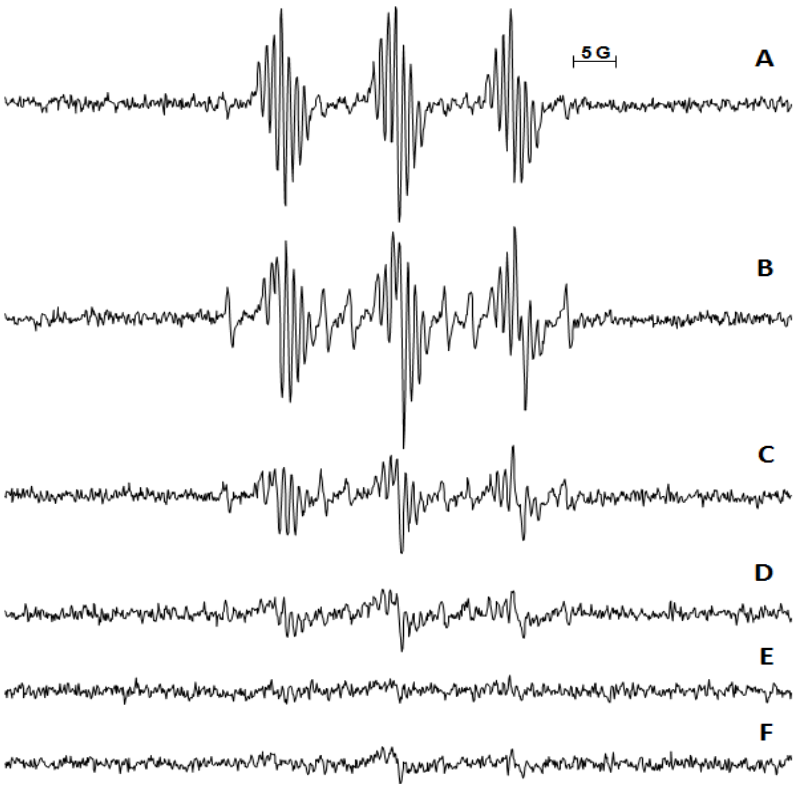


Figure 4.3 Attenuation of the ESR spectrum of AG-derived phenyl•/MNP by DHA

All experiments were carried out in Chelex-100-treated 0.1M PB pH 7.4, and spectra were recorded after a 5 minute incubation period after addition of the last reactant. The spectrum shown in (A) contained 14 mM MNP, 1 mM AG, 20 µM HRP, and 0.4 mM H2O2. The inclusion of 0.5 mM DHA (B), 2 mM DHA (C), 10 mM DHA (D), 25 mM DHA (E), and 50 mM DHA (F) were run under the same conditions. Instrument settings: Frequency: 9.79 GHz, power: 19.97 W, conversion time and time constant: 327.68 ms, scan time: 335.54 s, modulation amplitude: 0.4 G.

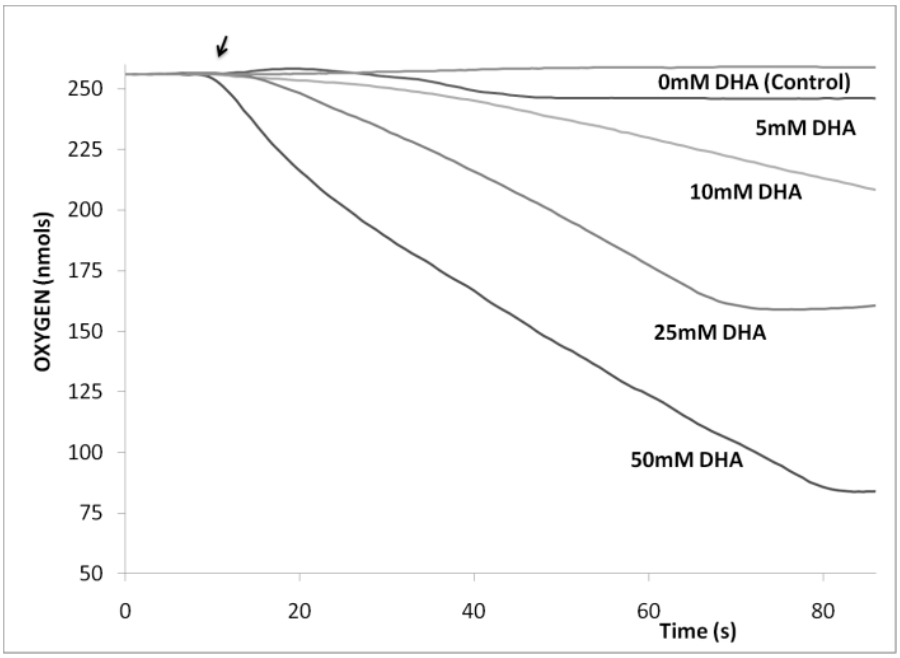


Figure 4.4 Dose dependency oxygen uptake curve of DHA at various concentrations with AG

Curve showing percentage of oxygen uptake vs time in seconds. 0 mM, 5 mM, 10 mM, 25 mM and 50 mM of DHA was used keeping AG 5 mM. 1 mM of H_2O_2 was used, and the reaction was initiated by adding 1 µM of HRP in phosphate buffer pH 7.4.

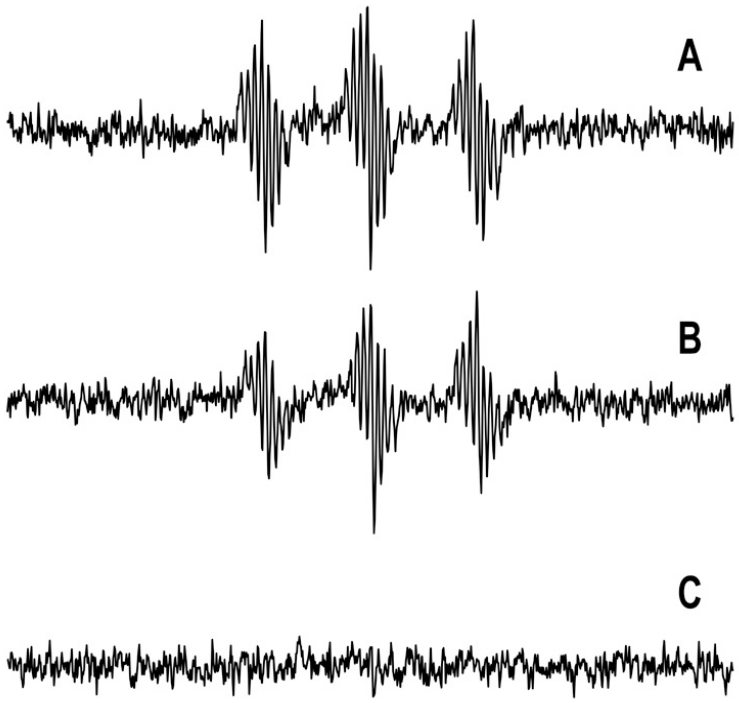


Figure 4.5 Comparative ESR spectrum of AG-derived phenyl•/MNP in presence of PUFA (sorbic acid),C and saturated fatty acid (hexanoic acid), B

ESR spectrum of AG-derived phenyl radical is abolished by the presence of sorbic acid, but not with hexanoic acid. The reaction was carried out in 0.1 M phosphate buffer (Chelex-100 treated, pH 7.4) containing 14 mM MNP, 1 mM AG, 20 μM HRP, and 0.4 mM H_2O_2 (A). When 5 mM hexanoic acid (the saturated derivative of sorbic acid) was added before initiating the reaction, there was some attenuation noted in the spectrum (B). The inclusion of 5 mM sorbic acid (C) abolished the spectrum in A. This indicates the requirement of unsaturated bonds in fatty acids for scavenging phenyl radicals. In addition, sorbic acid was soluble at the concentrations used.

4.3 Determination of phenyl radical induced lipid peroxidation product MDA by HPLC:

Our oxygen electrode data strongly suggests an oxidative reaction between the metabolically generated phenyl radical and unsaturated lipids. Further, EPR data suggests that oxidative reaction seen in oxygen electrode is due to direct interaction between the metabolically generated phenyl radicals and the investigated lipids. Lipid peroxidation and its attenuation due to presence of phenyl radical spin traps have been previously reported with phenylhydrazine (*54*). We implemented an HPLC methodology in conjunction with pre-column derivatization in order to detect MDA, a lipid peroxidation marker. We adapted our described HPLC assay with some modifications.

MDA-DNPH adduct has a chromatographic retention time of 10.5 min as shown in Figure 4.6. The peak volume at 10.5 min was collected and confirmed by MS/MS analysis in positive ionization mode, where MDA-DNPH pyrazole adduct appears at m/z 234.8 as shown in Figure 4.8. MDA-DNPH in the cyclized pyrazole form has been detected previously both *in vivo* and *in vitro* and our results from MS analysis of MDA-DNPH isolated by LC is in accordance with previous studies which detected and quantified MDA-DNPH as a pyrazole adduct (Figure 4.8) (*45, 55, 56*).

MDA produced from mild oxidation of sorbic acid according to a procedure described by Schmidt *et al.* using potassium dichromate and sulfuric acid was considered as a positive control and was also derivatised similarly by

DNPH, and the ether extract was analyzed by HPLC. Derivative peak was observed at t_R 10.6 min.

The reaction mixture prepared as described in chapter 3 containing sorbic acid, AG, HRP and peroxide was derivatized by DNPH and the extract was run in HPLC. Several peaks were obtained in the chromatographic run time, presumably of different lipid peroxidation products; however, a peak at 10.5 min was observed which correlates with the peak of MDA standard and with the peak obtained from the positive control reaction of sorbic acid (Figure 4.7) Necessary controls of the reaction mixture were also run, and no peaks appeared at the t_R of MDA-DNPH.

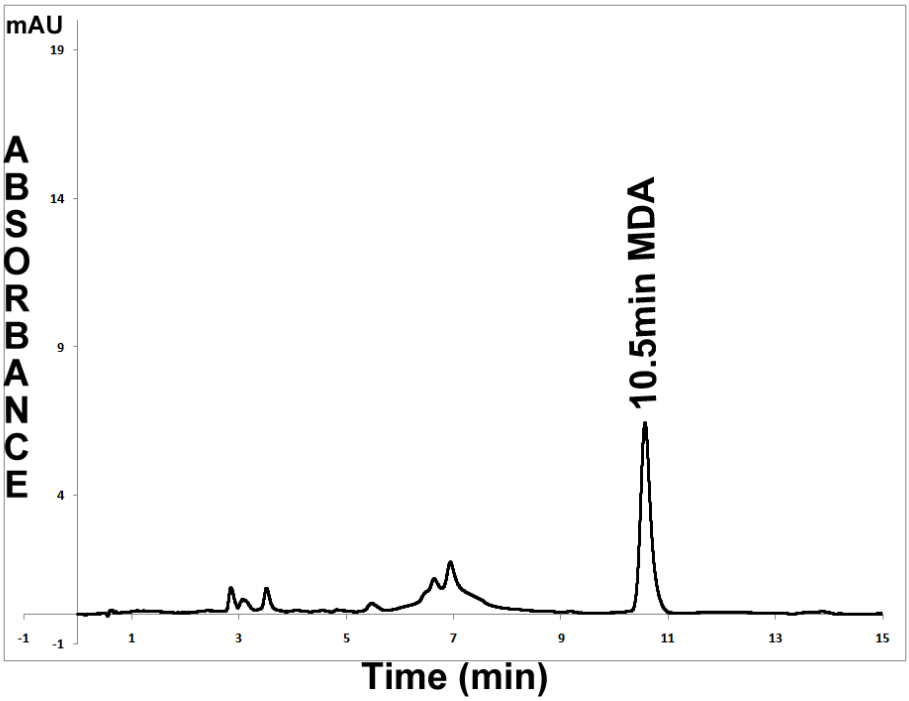


Figure 4.6 HPLC Chromatogram of MDA-DNPH derivative

Chromatogram showing MDA -DNPH derivative standard at retention time of 10.5 min. MDA was prepared from its stable precusore 1,1,3,3-tetramethoxypropane (938 µl) in acidic conditions (500 µl of 1M HCL) at room temperature. The MDA formed was neutralised with 1 N NaOH. The MDA standard thus prepared was derivatised with DNPH by adding 120 µl of MDA standard and 80 µl of 50 mM DNPH with acid catalysis. MDA-DNPH drevative was extracted with methyl-*t*-butyl ether and was subjected to solvent evaporation in gentle nitrogen stream. The dried extract was diluted in eluent prior to injection in HPLC.

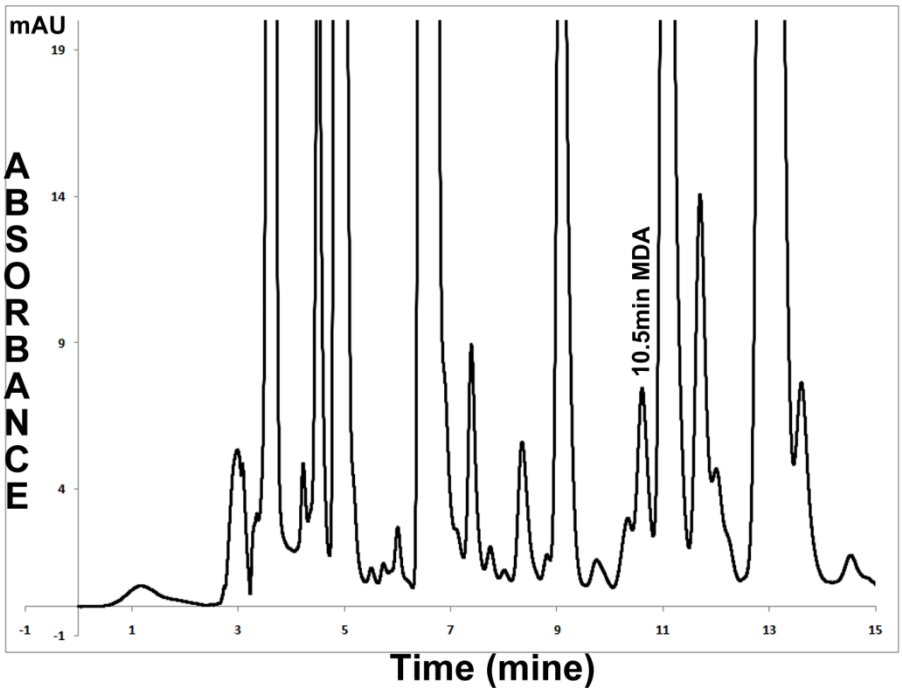


Figure 4.7 HPLC chromatogram of sorbic derived MDA

Chromatogram showing sorbic acid derived MDA-DNPH derivative at 10.5 min retention time. MDA was derived from mild acidic oxidation of sorbic acid in presence of 0.1 N potassium dichromate and sulfuric acid (0.1 N). The MDA obtained was derivatised with DNPH in similar conditions to that of MDA standard.

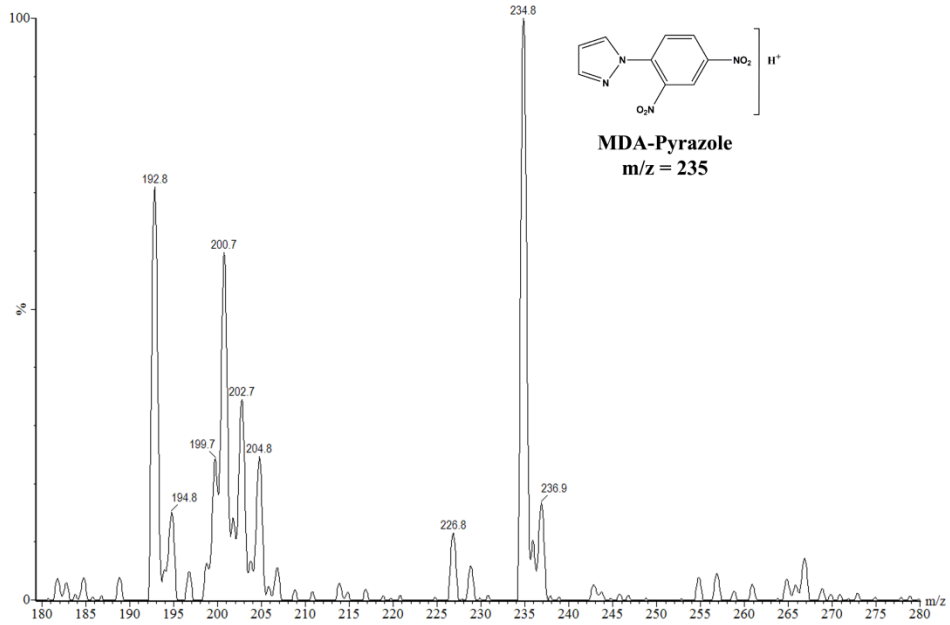


Figure 4.8 Mass spectrum of MDA peak collected from HPLC

MS peak at m/e 234.8 confirms the presence of MDA-DNPH pyrazole derivative MDA standard, peak collected at 10.5 min during HPLC analysis.

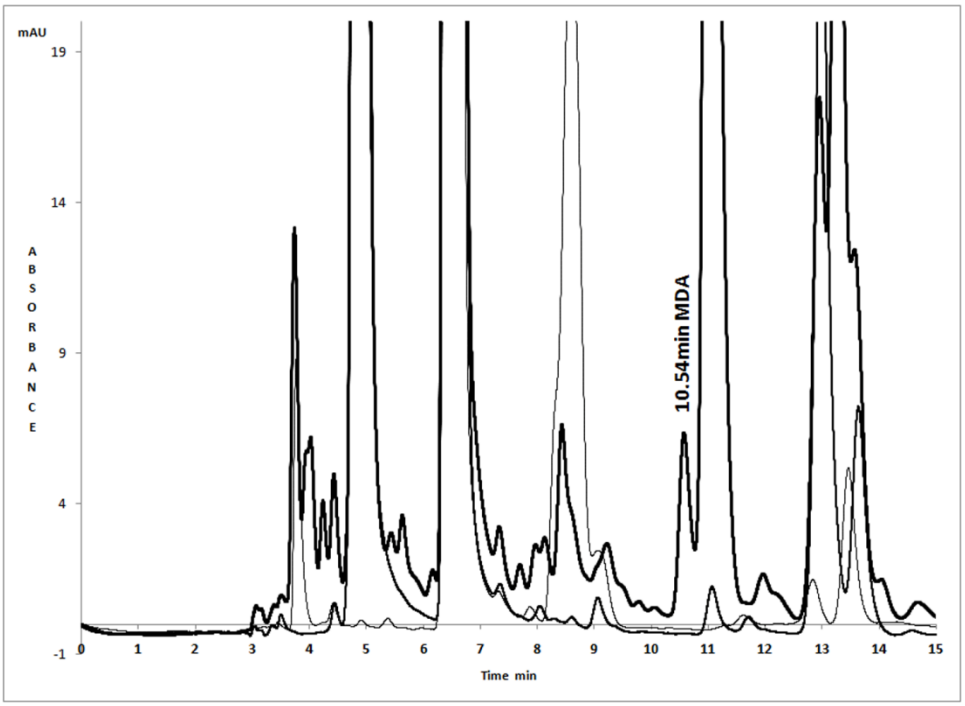


Figure 4.9 HPLC chromatogram of MDA formed by phenyl radical induce oxidation of sorbic acid

Chromatogram of MDA-DNPH pyrazole derivative formed during oxidation of sorbic acid by peroxidase derived phenyl radical from aminoglutethimide. Reaction mixture was prepared containing 20 mM of sorbic acid, 5mM of aminoglutethimide and 1 µM HRP/1 mM H_2O_2. The reaction was incubated at room tempurature for 30 min. The reaction was stopped by adding catalase and the reaction mixture was subjected to derivatisation with DNPH as discussed in figure 4.6.

4.4 Immuno-spin trapping experiments with HL-60 lysate:

In order to determine if the interaction of PUFAs with AG free radical metabolites could have potential cellular effects, we performed immuno-spin trapping experiments to determine if PUFAs could scavenge AG free radicals and would ultimately lead to the attenuation of protein radicals. It has been previously shown that AG produced protein radicals in HL-60 cells (*25*). We treated HL-60 cell lysate for 4 h with DMPO, AG, LA and initiated the reaction by adding glucose/GO (for H_2O_2 generation). The western blot was developed using anti-DMPO as shown in Figure 4.10. The greatest extent of anti-DMPO staining of protein bands were obtained from the lysate in lane 2 (complete system) which contained DMPO, AG, and glucose/GO. As indicated by the protein marker, the molecular weight of the most intense anti-DMPO immuno-reactive protein band (indicated by an asterisk) appeared at approximately 46 kDa. In addition, two faint bands appeared at ~90 kDa and ~60 kDa. The addition of LA to the complete system significantly attenuated anti-DMPO recognition (lane 3), and the absence of one reactant from the complete system prevented the detection of protein-DMPO adducts.

The same nitrocellulose membrane was stripped and reprobed with anti-MPO and anti-GAPDH (loading control) as shown in Figure 4.10. Two bands for MPO appeared in all lanes, which corresponded to the heavy chain of MPO (MPO-h, ~60 kDa) and pro-MPO (~90 kDa). A less intense band was stained at

a molecular weight of ~46 kDa (indicated by an asterisk). A single band at 37 kDa was obtained with anti-GAPDH, which used as a loading control.

We also determined the dose dependent effect of DHA on protein radical formation as described above with LA. In this instance, we used a lower concentration of AG (1 mM) and different concentrations of DHA (0.5 – 25 mM). In Figure 4.11 lane 1, two bands were detected with anti-DMPO (at ~46 kDa and ~60 kDa) in the reaction containing DMPO, AG, and glucose/GO, which was attenuated significantly by 25,10, and somewhat by 2 mM DHA. It appeared as though 2 mM DHA was the lowest concentration capable of attenuating protein radical formation, as 0.5 mM appeared to have no significant effect (lane 5). The absence of AG and presence of DHA in the reaction produced no anti-DMPO recognition (lane 6 and 7). These results were analogous to the findings in EPR spin trapping experiments with DHA (Figure 4.3). The loading control for this experiment showed that there was approximately equal loading of protein in each lane demonstrated with anti-MPO and anti-GAPDH as in Figure 4.11. Interestingly, we did not detect the MPO fragment found in Figure 4.10 in this instance.

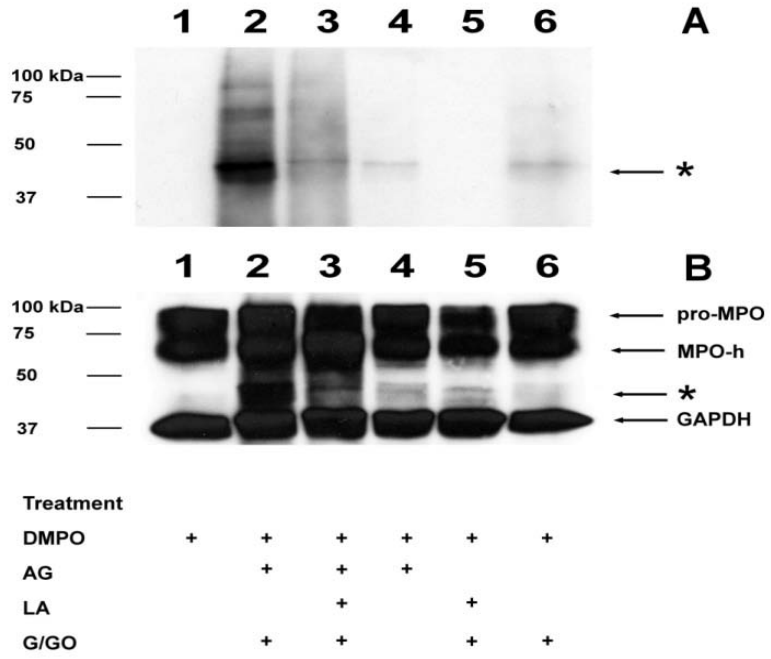


Figure 4.10 Phenyl radical induced protein radical attenuation by PUFA (LA)

LA attenuates aminoglutethimide (AG)-induced protein radical formation: HL-60 cell lysates were treated with DMPO (100 mM), AG (AG), 5 mM) and LA (100 mM) as indicated. The reaction was initiated by adding Glucose (5 mM) and GO (5 mU/100 µL). The reaction was incubated for 4 hrs at 25º C. Anti-DMPO detection of DMPO-protein adducts in the lysate is shown in (A). The nitrocellulose membrane shown in (B) was stripped and was incubated with anti-MPO and anti-GAPDH.

* Fragment of MPO detected by using anti-MPO (B), and immunoreactivity of a band with the same molecular weight with anti-DMPO (A). Heavy chain of MPO, MPO-h.

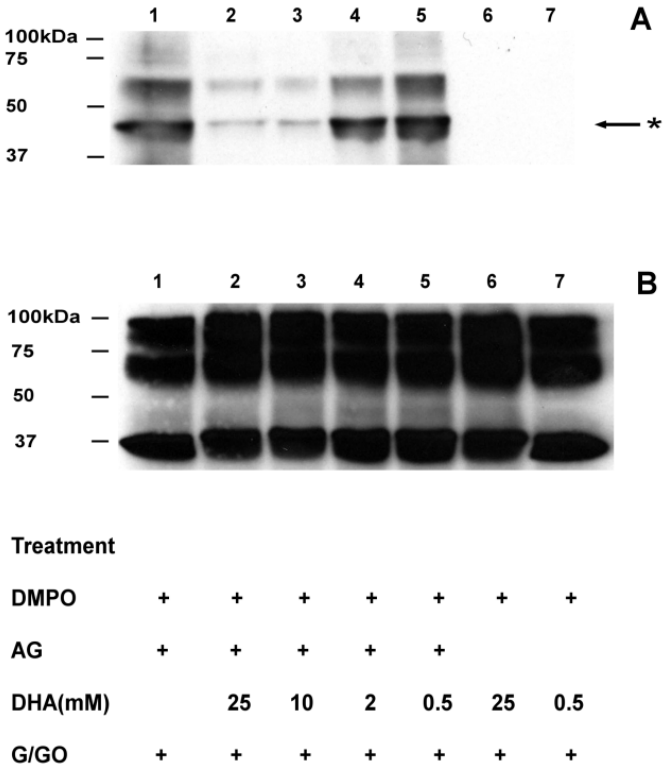


Figure 4.11 Dose dependent effect on protein radical attenuation by PUFA (DHA)

DHA dose-dependently attenuates AG-induced protein radical formation: HL-60 cell lysates were treated with DMPO (100 mM), AG (1 mM), and DHA (25 mM, 10 mM, 2 mM, and 0.5 mM) as indicated. The reaction in each lane was initiated by adding glucose (G, 5 mM) and GO (5 mU/100 μL). The reaction was incubated for 4 h at 25 °C. Anti-DMPO detection of MPO-protein adducts in the lysate is shown in A. The nitrocellulose membrane was stripped and reprobed with anti-MPO and anti- GAPDH (B).

4.5 DISCUSSION:

In this study, we have shown an apparent differential reactivity for the reaction of particular free radical metabolites of drugs and a series of congeners of fatty acid derivatives. Although the phenyl radical metabolite is a carbon centered radical, per se, its reactivity is different from other carbon centered free radical metabolites such as phenylbutazone carbon-centered free radicals (*57*) or carbon-centered lipid radicals; our observations with oxygen electrode experiments at room temperature suggest that phenyl radicals do not *apparently* consume oxygen in absence of PUFAs. Although many different reactions could be occurring, one possible explanation for this observation is due to the reversibility of the following reaction at temperatures lower than 450 K (*58*):

$R^{\bullet} + O_2 \leftrightarrow R\text{-}OO^{\bullet}$ (R = alkyl)

It is possible that the phenyl radicals produced in our experiments also reacted reversibly with O_2 in solution. The reaction of phenyl radicals with molecular oxygen will produce an energized phenyl-$OO^{\bullet}$ radical and lead to fragmentation products in a high temperature combustion environment (*59*), and at temperatures lower than 473 K in the gas phase (*60*). However, we have found that there is no detectable net change in the oxygen level of peroxidase-generated phenyl radicals run at room temperature in buffer. The high concentrations of fatty acids needed to react with phenyl radicals in our experiments may be due to overcoming a reversible addition-stabilization complex (*60*):

$phenyl^{\bullet} + O_2 \leftrightarrow phenyl\text{-}OO^{\bullet}$

Phenyl radicals have been previously synthesized by heating (60 °C) phenylazotriphenylmethane, which resulted in the formation of trityl radical, aryl (phenyl) radical, and N_2 (*61*). It was shown that phenyl radicals resulted in significant hydrogen abstraction with a thiophenol solvent, suggesting that thiyl radical products could form. In our study, we did not detect significant GSH oxidation with drugs that produced phenyl radicals. This could be due to using an order of magnitude less thiol (GSH) compared to studies carried out with thiophenol, or that the pKa of thiophenol (pKa = 6.6) made it a more favourable reactant than GSH (pKa = 9.2-9.42) (*62*, *63*).

One of the key findings of this study is the ability to distinguish the reactivity of aromatic amine free radical metabolites based on the targets which they react with. Previously, work from O'Brien's laboratory has shown that certain drugs can induce the peroxidation (oxygen consumption) of arachidonate (*27*). Among those drugs that were tested, 4-phenetidine (4-ethoxyaniline) showed the greatest extent of oxygen consumption. Interestingly, in this study we used *p*-toluidine (4-methylaniline) which also showed a significant rate of oxygen consumption in the presence of both GSH and LA. We found that compounds which induced preferential oxygen consumption of LA, but not GSH, appeared to correlate which the detection of phenyl radical formation in a HRP/H_2O_2 system (*11*). One exception that is puzzling has been procainamide (discussed below). The latter was shown to form a phenyl radical metabolite (*26*) but did not show any significant oxygen consumption with either LA or GSH. Moreover, procainamide was previously shown not to induce oxygen consumption in the

presence of GSH with HRP/H_2O_2; it was also inefficient in NADH oxidation (*36*). 2-Nitroaniline is a weak donor for the peroxidase cycle due to the electron withdrawing effects of the nitro group (Hammett sigma = 1.72) and close proximity to the amino group, which accounts for its insignificant activity in oxygen consumption with GSH and LA. Conversely, both *p*-anisidine and 3,4-dimethoxyaniline are potent peroxidase cycle donor substrates and effectively catalyzed GSH oxidation, but no oxidation of LA. These results are consistent with our hypothesis as no phenyl radical have been detected with these substrate. Although oxygen uptake (10±1 nmol/min) was observed by 3,4-dimethoxyanilne substrate with LA, it was an order of magnitude lower compared to other phenyl radical generating aromatic amine congeners. It is possible that 3,4-dimethoxyaniline produced phenyl radicals but were at a low level and hence, not detected.

In order to investigate the lack of expected oxygen consumption of LA induced by procainamide, we evaluated similar drug congeners. It appeared as though the amide substructure was associated with poor activity, since drugs containing esters or acids were more effective in catalyzing LA induced oxygen consumption. The triethylamine moiety appeared to also play a role in attenuating LA induced oxygen consumption since *p*-aminobenzamide showed oxygen consumption in contrast to procainamide. It is possible that the triethylamine substructure scavenged the phenyl radicals more favourably relative to LA. It has been shown that triethylamine scavenged phenolic radical cations at a rate constant of 10^9 mol^{-1} s^{-1} (*64*), and peroxy radicals (*65*). Also, neighbouring amide

groups in thioether derivatives appears to enhance their ease of oxidation since it was shown that the oxidation potential of such molecules was decreased (*66*). Taken together, the amide and triethylamine substructures may also scavenge phenyl radical metabolites generated in our experimental system.

We carried our studies further by evaluating the changes in oxygen consumption induced by incubation with different unsaturated compounds alkyl/fatty acids. When sorbic acid was used instead of LA, significantly higher oxygen consumption in the presence of an aniline derivative and HRP/H_2O_2 was observed. Sorbic acid, also known as 2,4-hexadienoic acid, possesses two unsaturated centres like LA; however, these are in conjugation. Conjugated double bonds are generally more reactive than their unconjugated counterparts which likely accounted for the enhanced response when using sorbic acid. DHA is reported to have dietary health benefits and is present in fish oil (*67*) and showed greater oxygen consumption than LA; this was likely due to the increased number of double bonds in its structure, although this increase was not proportional to the number of double bonds. This implies that DHA could have more potent phenyl radical scavenging activity than LA. The necessity of possessing a double bond in the alkyl/fatty acid as a requirement for oxygen consumption was shown by the lack of activity of hexanoic acid,the saturated analogue of sorbic acid. In addition, possessing only one double bond did not appear to result in significant reactivity with phenyl radical metabolites as the oxygen consumption with acrylic acid (prop-2-eneoic acid) was not significantly different from propionic acid.

In order to obtain direct evidence that LA was reacting with the phenyl radical metabolite, we performed EPR spin-trapping experiments. These experiments were designed to detect phenyl radical-spin trap adducts, and evaluate the effect of fatty acids in this reaction. We performed experiments with MNP which confirmed (based on splitting constants) previous findings for an AG-derived phenyl-MNP radical adduct (*25*). The free radical scavenging property of conjugated linoleic acid has been shown previously (*32, 37*). More importantly, we demonstrated the attenuation of phenyl radical metabolite detection in the presence of LA or DHA. The dose dependent effect from DHA-mediated attenuation of the phenyl radical spectrum suggests that it effectively reacted with phenyl radicals. The findings from the ESR were reflected by similar dose-dependent oxygen consumption experiments, but appeared to highlight that the reactions in the ESR were more sensitive compared to oxygen consumption. Furthermore, we expected to detect a secondary spin adduct arising from LA or DHA carbon-centered or peroxyl radicals since phenyl radical scavenging together with oxygen consumption would require obligate formation of such secondary radicals. Spin adducts of DMPO$^{\bullet}$/O-R or DMPO$^{\bullet}$/OO-R (R = PUFA) have been carefully characterized previously by others (*68*). It has also been shown that LA hydroperoxide is degraded in the presence of HRP and donor substrates (*69*). Therefore, the presence of HRP in our system may have induced the breakdown of PUFA hydroperoxides and potentially LA$^{\bullet}$/MNP or DHA$^{\bullet}$/MNP spin adducts to EPR silent species. Interestingly, experiments with DHA showed that at low concentrations of DHA, a carbon-centered radical with two equivalent

hydrogens was detected. This has been previously assigned to $^{\bullet}CH_2OH$; however, all of our experiments used equivalent concentrations of methanol. It is possible that this radical may have formed by the oxidation of DHA to an alkoxyl radical formed upon reacting with phenyl radicals.

Our oxygen electrode studies and ESR studies suggest the possibility of phenyl radical mediated lipid oxidation. Often, lipid oxidation leads to formation of lipid peroxidation products which further breakdown to produce malondialdehyde (MDA) and hydroxynonenal compounds. In order to determine the lipid peroxidation product, MDA, we used HPLC. MDA can be derivatized using DNPH into a stable pyrazole complex which can be separated and detected by liquid chromatography. The MDA-DNPH pyrazole is reported to possess an absorbance in the range of 310 nm (*44, 70*). The ether extract of MDA-DNPH derivative was dissolved in the mobile phase & directly injected into the chromatograph. The peak volume obtained at t_R 10.5 min (Figure 4.6) was collected and was subjected to mass analysis. Mass spectrum of the peak obtained in the positive ion mode confirmed the formation of MDA-DNPH pyrazole with an m/z of 234.8 (Figure 4.8). The MS analysis and the overall spectra also correlates with previous studies performed in order to elucidate the chemical nature of MDA-DNPH complex (*55, 56*). Furthermore, MDA derived from mild oxidation of sorbic acid in presence of potassium dichromate in acidic conditions was likewise analysed as MDA standard. The appearance of a peak at t_R 10.5 min (Figure 4.7) correlates with the standard MDA peak and confirms the presence of sorbic acid-derived MDA. In order to detect the MDA formation due to

metabolically generated phenyl radical, we performed the reaction using aminoglutethimide and sorbic acid in presence of HRP and H_2O_2 as described in Table 4.3. The derivatized reaction mixture was extracted with methy-*t*-butyl ether and was dissolved in the mobile phase prior to injection into the system. Any oxidation of PUFA due to metabolically generated phenyl radicals should result in the formation of MDA and the subsequent appearance of a peak at the same retention time as the standard or the positive control. The HPLC results obtained were as anticipated and lipid peroxidation was confirmed by identifying the MDA-DNPH pyrazole peak at t_R 10.5 min. Two negative controls were prepared one in the absence of drug and other in the absence of PUFA . These did not result in any elution of peaks at the characteristic retention time of MDA derivative. This is in agreement with our oxygen electrode and ESR data which shows absence of any oxidative reaction when one of these reactants are absent. In the HPLC chromatogram there appears to be other peaks whose identity was not confirmed, especially in the chromatogram obtained with mild acidic oxidation of sorbic acid by potassium dichromate. It is conceivable that these might be other oxidation products of sorbic acid. It is known that sorbic acid gives rise to various oxidation products like acetaldehyde, fumaraldehyde, acetic acid, fumaric acid, and polymeric products (*71*). Hence, we suggest that apart from MDA there is a high possibility of formation of multiple oxidative products of PUFA induced due to metabolically generated phenyl radical.

It has been previously shown that there appears to be a correlation between aromatic amines which form phenyl radical metabolites with protein

radical formation (*11*). Since we have determined in this study that LA appears to scavenge phenyl radical metabolites, we tested the ability of LA to prevent protein radical formation. Previously, it has been shown that AG is metabolized by MPO in HL-60 cells to a phenyl radical, which is thought to convert the metabolizing enzyme (MPO itself) into a protein radical (*25*). We used HL-60 cell lysate to detect AG-induced protein radical formation and evaluated the effect of LA. Interestingly, when cell lysate was treated with DMPO, AG and glucose/GO, the western blot results showed the protein–DMPO adduct at molecular weight of ~46 kDa, while the molecular weight of the major protein-DMPO radical adduct from intact cells was detected at ~60 kDa (*25*). The protein-DMPO adduct appears at lower molecular weight (~46 kDa) than that of the heavy chain of MPO (MPO-h, ~60 kDa) which we believe represents a fragmentation product (Figure 4.10). This was further confirmed when this fragment was detected with anti-MPO. The fragments of MPO have been reported previously by others (*72, 73*), and the molecular weights of MPO fragments (40 k – 46 kDa) detected previously are in accordance with our results. Thus, it is possible that the phenyl radical metabolite generated from AG might also be responsible for causing fragmentation of MPO along with converting it into a protein radical. Interestingly, when lower AG concentrations were used either with lysate the putative MPO fragment was not detected, suggesting that high concentrations of AG free radicals may be required to catalyze protein scission (Figure 4.11). Since no DMPO-protein adduct was detected on the light chain of MPO (~14 kDa), this suggests that only heavy chain of the enzyme is involved in reaction with phenyl

radical and thus in protein radical formation. Furthermore, when LA was introduced in the cell lysate reaction mixture, it attenuated the MPO radical formation. Similar results were obtained when DHA was used in place of LA. Moreover, we confirmed a dose dependent attenuation of protein radical with PUFA (DHA) which suggests that phenyl radical is capable of causing oxidation of both PUFAs and protein at the same time if both area available Figure 4.11. However, PUFAs become predominating targets of phenyl radical when present in excess. These results are in accordance with the results of EPR, where LA plays a role of scavenging the AG phenyl radical which in turn should prevent the phenyl radical and MPO interaction thus inhibiting formation of protein (MPO) radical.

4.6 Conclusion:

The high concentration of AG, and PUFAs used are not achievable *in vivo*, and the level of H_2O_2 generated by GO (5 µmol/min) is greater than would be expected *in vivo*. Although we used lower concentrations of AG to generate protein radicals and demonstrated the attenuating effect of DHA, this study was constrained by limits of detection. However, it can be concluded that excess concentrations of certain PUFAs appear to be preferential targets of metabolically generated phenyl radicals from AG, over MPO. At the *in vivo* level, such a high concentration of one particular PUFA is highly unlikely; but it is possible that a combination of different fatty acids could result in reacting with drug-derived

phenyl radicals. However, from our findings it can be concluded that the selectivity of either of these target molecules depends on the relative availability of these targets. If a cumulative concentration of PUFAs were to be present in the same compartment as phenyl radicals are formed, the consequences would likely lead to lipid peroxidation induced toxicity.

Further, as the aromatic amines and their peroxidase (myeloperoxidase or HRP) mediated metabolism is associated with drug induced agranulocytosis toxicity (*11, 25, 26*) hence, these studies can be extrapolated to give a new approach of mechanism of aromatic amine induced agranulocytosis toxicity.

4.7 Future studies:

In these studies, *in vitro* studies were done using HL-60 cell lysate which are rich in myeloperoxidase and resemble precursors of neutrophils. Also, phenyl radicals have been detected in enzymatic system and but not intracellularly. The formation of free radical metabolites from aromatic amine metabolism in cells containing myeloperoxidase (neutrophil or HL-60) is expected since isolated myeloperoxidase catalyzed metabolite formation. However, the quantity of phenyl radical metabolite produced intracellularly remains to be established since there are many factors (e.g., ascorbic acid) that can influence the yield of phenyl radical metabolites. Studies can be carried out in human neutrophils to detect the generation of phenyl radicals inside the neutrophils and lipid peroxidation as a consequence of their formation. This can be achieved by doing the studies on

isolated neutrophils in presence of suitable non-toxic spin-trapping agents (*viz*. DMPO or PBN) which can trap the free radical metabolites of aromatic amines and can be analyzed in ESR or HPLC. However, formation of various intracellular free radical products produced during redox reactions can be anticipated to cause interference during these studies. Further, lipid peroxidation can be determined by assaying for intracellular markers like MDA or isoprostanes (marker for free radical mediated lipid peroxidation generated from arachidonates and docosahexaenoates). Further, hydroxynonanal (HNE) is another stable product of lipid peroxidation which can be detected similarly as MDA intracellularly. These studies suggest that, conceptually the formation of protein radical and lipid peroxidation induced by aromatic amine free radical metabolites can be a possible toxicity mechanism of drug induced agranulocytosis. However, until now there has been no direct evidence for correlation of these findings with agranulocytosis toxicity is been presented. Further studies need to be carried out both *in vitro* and *in vivo*, in order to prove the direct association of agranulocytosis with the free radical metabolites of aromatic amines during peroxidase metabolism and their reactivity towards above suggested cellular targets (protein, GSH and PUFAs). It could be possible to assay F_2-isoprostane levels *in vivo* (biomarkers of lipid peroxidation) induced by phenyl radical metabolites of aromatic amine drugs. Hydroxy octadecadienoic acid, as another lipid oxidation marker formed due to oxidation of linoleates can also be investigated *in vivo*.

Further research is required to validate the formation of aromatic amine derived free radical metabolites *in vivo* in the tissues containing myeloperoxidase enzyme. Also, *in vitro* and *in vivo* studies are needed to find possible cellular targets of above free radical metabolites of aromatic amines other than proteins, GSH and PUFAs.

4.8 REFERENCES:

(1) Siraki, A. G., Chan, T. S., Galati, G., Teng, S., and O'Brien, P. J. (2002) N-oxidation of aromatic amines by intracellular oxidases. *Drug Metab Rev*, *34*, 549-564.

(2) Kato, R. (1986) Metabolic activation of mutagenic heterocyclic aromatic amines from protein pyrolysates. *Crit Rev Toxicol*, *16*, 307-348.

(3) Miller, J. A. (1998) The metabolism of xenobiotics to reactive electrophiles in chemical carcinogenesis and mutagenesis: A collaboration with Elizabeth Cavert Miller and our associates. *Drug Metabolism Reviews*, *30*, 645-674.

(4) Miller, J. A., and Miller, E. C. (1948) The carcinogenicity of certain derivatives of p-dimethylaminozobenz in. *The Journal of experimental medicine*, *87*, 139-156.

(5) Lin, J. K., Miller, J. A., and Miller, E. C. (1968) Studies on structures of polar dyes derived from the liver proteins of rats fed N-methyl-4-aminoazobenzene. II. Identity of synthetic 3-(homocystein-S-yl)-N-methyl-4-aminoazobenzene with the major polar dye P2b. *Biochemistry*, *7*, 1889-1895.

(6) Weisburger, J. H. (2002) Comments on the history and importance of aromatic and heterocyclic amines in public health. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *506-507*, 9-20.

(7) Tannenbaum, S. R., and Skipper, P. L. (1994) [40] Quantitative analysis of hemoglobin-xenobiotic adducts, In *Methods in Enzymology* (Johannes Everse, K. D. V. R. M. W., Ed.) pp 625-632, Academic Press.

(8) Kiese, M. (1966) The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. *Pharmacological Reviews*, *18*, 1091-1161.

(9) Uetrecht, J. P. (1989) Idiosyncratic Drug Reactions: Possible Role of Reactive Metabolites Generated by Leukocytes. *Pharmaceutical Research*, *6*, 265-273.

(10) Uetrecht, J. P. (1992) The role of leukocyte-generated reactive metabolities in the pathogenesis of idiosyncratic drug reactions. *Drug Metabolism Reviews*, *24*, 299-366.

(11) Siraki, A. G., Jiang, J., and Mason, R. P. (2010) Investigating the mechanisms of aromatic amine-induced protein free radical formation by quantitative structure-activity relationships: implications for drug-induced agranulocytosis. *Chem Res Toxicol*, *23*, 880-887.

(12) Andrès, E., Zimmer, J., Affenberger, S., Federici, L., Alt, M., and Maloisel, F. (2006) Idiosyncratic drug-induced agranulocytosis: Update of an old disorder. *European Journal of Internal Medicine, 17*, 529-535.

(13) Uetrecht, J. (2007) Idiosyncratic drug reactions: current understanding. *Annu Rev Pharmacol Toxicol*, *47*, 513-539.

(14) Andrès, E., Noel, E., Kurtz, J. E., Loukili, N. H., Kaltenbach, G., and Maloisel, F. (2004) Life-threatening idiosyncratic drug-induced agranulocytosis in elderly patients. *Drugs and Aging*, *21*, 427-435.

(15) John E Godwin, C. D. B., Kush Sachdeva. (2011) Neutropenia *http://emedicine.medscape.com*.

(16) Sprikkelman, A., De Wolf, J. T. M., and Vellenga, E. (1994) The application of hematopoietic growth factors in drug-induced agranuiocytosis: A review of 70 cases. *Leukemia*, *8*, 2031-2036.

(17) Andrès, E., Maloisel, F., Kurtz, J.-E., Kaltenbach, G., Alt, M., Weber, J.-C., Sibilia, J., Schlienger, J.-L., Blicklé, J.-F., Brogard, J.-M., and Dufour, P. (2002) Modern management of non-chemotherapy drug-induced agranulocytosis: a monocentric cohort study of 90 cases and review of the literature. *European Journal of Internal Medicine*, *13*, 324-328.

(18) Cramer, J. W., Miller, J. A., and Miller, E. C. (1960) N-Hydroxylation: A new metabolic reaction observed in the rat with the carcinogen 2-acetylaminofluorene. *The Journal of biological chemistry*, *235*, 885-888.

(19) Rabstein, S., Brüning, T., Harth, V., Fischer, H. P., Haas, S., Weiss, T., Spickenheuer, A., Pierl, C., Justenhoven, C., Illig, T., Vollmert, C., Baisch, C., Ko, Y. D., Hamann, U., Brauch, H., and Pesch, B. (2010) N-acetyltransferase 2, exposure to aromatic and heterocyclic amines, and receptor-defined breast cancer. *European Journal of Cancer Prevention*, *19*, 100-109.

(20) Gorrod, J. W., and Manson, D. (1986) The metabolism of aromatic amines. *Xenobiotica*, *16*, 933-955.

(21) Hjelm, M., and De Verdier, C. H. (1965) Biochemical effects of aromatic amines-I. Methaemoglobinaemia, haemolysis and heinz-body formation induced by 4,4'-diaminodiphenylsulphone. *Biochemical Pharmacology*, *14*, 1119-1128.

(22) Siraki, A. G., Deterding, L. J., Bonini, M. G., Jiang, J., Ehrenshaft, M., Tomer, K. B., and Mason, R. P. (2008) Procainamide, but not N-acetylprocainamide, induces protein free radical formation on myeloperoxidase: a potential mechanism of agranulocytosis. *Chem Res Toxicol*, *21*, 1143-1153.

(23) Eyer, P. (1988) Detoxication of N-Oxygenated Arylamines in Erythrocytes. an Overview. *Xenobiotica*, *18*, 1327-1333.

(24) Fischer, V., Haar, J. A., Greiner, L., Lloyd, R. V., and Mason, R. P. (1991) Possible role of free radical formation in clozapine (clozaril)-induced agranulocytosis. *Molecular Pharmacology, 40*, 846-853.

(25) Siraki, A. G., Bonini, M. G., Jiang, J., Ehrenshaft, M., and Mason, R. P. (2007) Aminoglutethimide-induced protein free radical formation on myeloperoxidase: a potential mechanism of agranulocytosis. *Chem Res Toxicol, 20*, 1038-1045.

(26) Siraki, A. G., Deterding, L. J., Bonini, M. G., Jiang, J., Ehrenshaft, M., Tomer, K. B., and Mason, R. P. (2008) Procainamide, but not N-acetylprocainamide, induces protein free radical formation on myeloperoxidase: A potential mechanism of agranulocytosis. *Chemical Research in Toxicology, 21*, 1143-1153.

(27) Subrahmanyam, V. V., and O'Brien, P. J. (1985) Peroxidase catalysed oxygen activation by arylamine carcinogens and phenol. *Chemico-Biological Interactions, 56*, 185-199.

(28) Tsuruta, Y., Subrahmanyam, V. V., Marshall, W., and O'Brien, P. J. (1985) Peroxidase-mediated irreversible binding of arylamine carcinogens to DNA in intact polymorphonuclear leukocytes activated by a tumor promoter. *Chemico-Biological Interactions, 53*, 25-35.

(29) Subrahmanyam, V. V., McGirr, L. G., and O'Brien, P. J. (1987) Glutathione oxidation during peroxidase catalysed drug metabolism. *Chemico-Biological Interactions, 61*, 45-59.

(30) Stohs, S. J. (1995) The role of free radicals in toxicity and disease. *Journal of Basic Clinical Physiology and Pharmacology, 6*, 205-228.

(31) Docampo, R., and Moreno, S. N. J. (1984) Free Radical Metabolites in the Mode of Action of Chemotherapeutic Agents and Phagocytic Cells on Trypanosoma cruzi. *Reviews of Infectious Diseases, 6*, 223-238.

(32) Yu, L., Adams, D., and Gabel, M. (2002) Conjugated Linoleic Acid Isomers Differ in Their Free Radical Scavenging Properties. *Journal of Agricultural and Food Chemistry, 50*, 4135-4140.

(33) Trush, M. A., Mimnaugh, E. G., and Gram, T. E. (1982) Activation of pharmacologic agents to radical intermediates. Implications for the role of free radicals in drug action and toxicity. *Biochemical Pharmacology, 31*, 3335-3346.

(34) Pastore, A., Federici, G., Bertini, E., and Piemonte, F. (2003) Analysis of glutathione: Implication in redox and detoxification. *Clinica Chimica Acta, 333*, 19-39.

(35) Subrahmanyam, V. V., McGirr, L. G., and O'Brien, P. J. (1987) Oxygen activation during drug metabolism. *Pharmacology and Therpeutics, 33*, 63-72.

(36) Galati, G., Tafazoli, S., Sabzevari, O., Chan, T. S., and O'Brien, P. J. (2002) Idiosyncratic NSAID drug induced oxidative stress. *Chemico-Biological Interactions*, *142*, 25-41.

(37) Yu, L. (2001) Free Radical Scavenging Properties of Conjugated Linoleic Acids. *Journal of Agricultural and Food Chemistry*, *49*, 3452-3456.

(38) Ramamoorthy, R., Dutta, P. K., and Akbar, S. A. (2003) Oxygen sensors: Materials, methods, designs and applications. *Journal of Materials Science*, *38*, 4271-4282.

(39) Intitute of Nutritional and Physiological Chemistry, U. o. V. http://ipm.univie.ac.at/en/institute-of-nutritional-and-physiological-chemistry/toolbox/bioactive-analysis/clark-electrode/

(40) http://www.eutechinst.com/. http://www.eutechinst.com/techtips/tech-tips16.htm.

(41) Kempe, S., Metz, H., and Mäder, K. (2010) Application of Electron Paramagnetic Resonance (EPR) spectroscopy and imaging in drug delivery research - Chances and challenges. *European Journal of Pharmaceutics and Biopharmaceutics*, *74*, 55-66.

(42) Rhodes, C. J. (2005) ELECTRON SPIN RESONANCE SPECTROSCOPY | Principles and Instrumentation, In *Encyclopedia of Analytical Science* (Paul, W., Alan, T., and Colin, P., Eds.) pp 332-337, Elsevier, Oxford.

(43) Rhodes, C. J., and Morris, H. (2005) ELECTRON SPIN RESONANCE SPECTROSCOPY | Specialized Techniques, In *Encyclopedia of Analytical Science* (Paul, W., Alan, T., and Colin, P., Eds.) pp 337-343, Elsevier, Oxford.

(44) Mendes, R., Cardoso, C., and Pestana, C. (2009) Measurement of malondialdehyde in fish: A comparison study between HPLC methods and the traditional spectrophotometric test. *Food Chemistry*, *112*, 1038-1045.

(45) Andreoli, R., Manini, P., Corradi, M., Mutti, A., and Niessen, W. M. A. (2003) Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, *17*, 637-645.

(46) Trivella, A., Coussan, S., and Chiavassa, T. (2008) Malonaldehyde synthesis. *Synthetic Communications*, *38*, 3285-3290.

(47) Michail, K., Matzi, V., Maier, A., Herwig, R., Greilberger, J., Juan, H., Kunert, O., and Wintersteiger, R. (2007) Hydroxymethylfurfural: An enemy or a friendly xenobiotic? A bioanalytical approach. *Analytical and Bioanalytical Chemistry*, *387*, 2801-2814.

(48) Michail, K., Juan, H., Maier, A., Matzi, V., Greilberger, J., and Wintersteiger, R. (2007) Development and validation of a liquid chromatographic method for the determination of hydroxymethylfurfural and alpha-ketoglutaric acid in human plasma. *Analytica Chimica Acta, 581*, 287-297.

(49) Gutfinger, T., Ashkenazy, R., and Letan, A. (1976) Determination of benzoic and sorbic acids in orange juice. *Analyst (London), 101*, 49-54.

(50) Van Gend, H. W. (1973) Automated colorimetric determination of sorbic acid after continuous separation by volatilization. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A, 151*, 81-83.

(51) Davidek, J., and Frimlova, E. (1964) Colorimetric determination of sorbic acid with 2-thiobarbituric acid. *Sb. Vysoke Skoly Chem.Technol. Praze, Potravinarska Technol., 6*, 47-63.

(52) Schmidt, H. (1960) Eine spezifische colorimetrische Methode zur Bestimmung der Sorbinsäure. *Fresenius' Journal of Analytical Chemistry, 178*, 173-184.

(53) Subrahmanyam, V. V., McGirr, L. G., and O'Brien, P. J. (1987) Oxygen activation during drug metabolism. *Pharmacology and Therapeutics, 33*, 63-72.

(54) Hill, H. A. O., and Thornalley, P. J. (1983) The effect of spin traps on phenylhydrazine-induced haemolysis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 762*, 44-51.

(55) Cordis, G. A., Maulik, N., and Das, D. K. (1995) Detection of oxidative stress in heart by estimating the dinitrophenylhydrazine derivative of malonaldehyde. *Journal of Molecular and Cellular Cardiology, 27*, 1645-1653.

(56) Fenaille, F., Mottier, P., Turesky, R. J., Ali, S., and Guy, P. A. (2001) Comparison of analytical techniques to quantify malondialdehyde in milk powders. *Journal of Chromatography A, 921*, 237-245.

(57) Hughes, M. F., Mason, R. P., and Eling, T. E. (1988) Prostaglandin hydroperoxidase-dependent oxidation of phenylbutazone: Relationship to inhibition of prostaglandin cyclooxygenase. *Molecular Pharmacology, 34*, 186-193.

(58) Benson, S. W. (1996) Some observations on the thermochemistry and kinetics of peroxy radicals. *Journal of Physical Chemistry, 100*, 13544-13547.

(59) Sebbar, N., Bockhorn, H., and Bozzelli, J. (2008) Thermodynamic properties of the species resulting from the phenyl radical with O2 reaction system. *International Journal of Chemical Kinetics, 40*, 583-604.

(60) Yu, T., and Lin, M. C. (1994) Kinetics of the C6H5 + O2 reaction at low temperatures. *Journal of the American Chemical Society*, *116*, 9571-9576.

(61) Pryor, W. A., Echols Jr, J. T., and Smith, K. (1966) Rates of the reactions of substituted phenyl radicals with hydrogen donors. *Journal of the American Chemical Society*, *88*, 1189-1199.

(62) Kreevoy, M. M., Harper, E. T., Duvall, R. E., Wilgus Iii, H. S., and Ditsch, L. T. (1960) Inductive effects on the acid dissociation constants of mercaptans. *Journal of the American Chemical Society*, *82*, 4899-4902.

(63) Tang, S. S., and Chang, G. G. (1996) Kinetic characterization of the endogenous glutathione transferase activity of octopus lens S-crystallin. *Journal of Biochemistry*, *119*, 1182-1188.

(64) Joshi, R., Naumov, S., Kapoor, S., Mukherjee, T., Hermann, R., and Brede, O. (2004) Phenol radical cations and phenoxyl radicals in electron transfer from the natural phenols sesamol, curcumin and trolox to the parent radical cations of 1-chlorobutane. *Journal of Physical Organic Chemistry*, *17*, 665-674.

(65) Görner, H. (2009) Photoinduced oxygen uptake for benzoylpyridines and dipyridylketones in aqueous solution. *Journal of Photochemistry and Photobiology A: Chemistry*, *208*, 141-146.

(66) Glass, R. S., Hug, G. L., Schöneich, C., Wilson, G. S., Kuznetsova, L., Lee, T. M., Ammam, M., Lorance, E., Nauser, T., Nichol, G. S., and Yamamoto, T. (2009) Neighboring amide participation in thioether oxidation: Relevance to biological oxidation. *Journal of the American Chemical Society*, *131*, 13791-13805.

(67) Simopoulos, A. P. (1991) Omega-3 fatty acids in health and disease and in growth and development. *American Journal of Clinical Nutrition*, *54*, 438-463.

(68) Dikalov, S. I., and Mason, R. P. (2001) Spin trapping of polyunsaturated fatty acid-derived peroxyl radicals: Reassignment to alkoxyl radical adducts. *Free Radical Biology and Medicine*, *30*, 187-197.

(69) Shirasaka, N., Ohnishi, H., Sato, K., Miyamoto, R., Terashita, T., and Yoshizumi, H. (2005) Horseradish peroxidase degrades lipid hydroperoxides and suppresses lipid peroxidation of polyunsaturated fatty acids in the presence of phenolic antioxidants. *Journal of Bioscience and Bioengineering*, *100*, 653-656.

(70) Mateos, R., Lecumberri, E., Ramos, S., Goya, L., and Bravo, L. (2005) Determination of malondialdehyde (MDA) by high-performance liquid chromatography in serum and liver as a biomarker for oxidative stress: Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants from fruits. *Journal of*

Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, *827*, 76-82.

(71) Lück, E., Jager, M., and Raczek, N. (2000) Sorbic Acid, In *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA.

(72) Otani, I. (1997) Myeloperoxidase fragments reacting with serum from a patient with myeloperoxidase-anti-neutrophil cytoplasmic antibody-positive glomerulonephritis. *Journal of Infection and Chemotherapy*, *3*, 164-169.

(73) Lemansky, P., Gerecitano-Schmidek, M., Das, R. C., Schmidt, B., and Hasilik, A. (2003) Targeting myeloperoxidase to azurophilic granules in HL-60 cells. *Journal of Leukocyte Biology*, *74*, 542-550.